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(54) Title: TOPICAL COMPOSITION CONTAINING HYALURONIC ACID AND NSAIDS

(57) Abstract

A pharmaceutical composition comprising a plurality of effective non-toxic dosage amounts of a composition for topical administration to the site of pathology and/or trauma of skin and/or exposed tissue of a human patient in need of treatment suffering from a disease or condition, each such dosage amount comprising a therapeutically effective non-toxic (to the patient) dosage amount of a drug for the treatment of the disease and/or condition of the skin and/or exposed tissue at the site of the pathology and/or trauma and an effective non-toxic dosage amount of hyaluronic acid and/or salts thereof and/or homologues, analogues, derivatives, complexes, esters, fragments, and/or sub-units of hyaluronic acid to transport (to facilitate or cause the transport of) the drug to the site of the pathology and/or trauma of the disease or condition.

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TOPICAL COMPOSITION CONTAINING HYALURONIC ACID AND NSAIDS

FIELD OF INVENTION

This invention relates to the treatment of disease and conditions of the skin and exposed tissue. In some embodiments this invention finds application to the treatment of a disease or condition of the skin and exposed tissue including basal cell carcinoma, squamous cell tumours, metastatic cancer of the breast to the skin, primary and metastatic melanoma in the skin, malignancies and tumours in the skin, genital warts (condyloma acuminata), cervical cancer, HPV (Human Papilloma Virus) including HPV (Human Papilloma Virus) on the cervix, psoriasis (both plaque-type psoriasis and nail bed psoriasis), corns on the feet, actinic keratoses lesions, "liver" spots, fungal lesions, and other such types of lesions, and hair loss on the head of a pregnant women.

This invention also relates to compositions and formulations suitable for use in such treatments, the use of such formulations in such treatments, methods of such treatment, and the delivery of drugs for such treatments.

BACKGROUND OF THE INVENTION

Basal cell carcinoma is presently treated by surgery. Each lesion, together with all surrounding and underlying tissue (dermis, epidermis, and subdermis), is cut out. In some instances, surgery, while necessary for the patient's welfare, puts the patient at risk in some other respect (for example, the removal of a lesion on a patient's temple (resection) may jeopardize the patient's health). Squamous cell tumours are also treated the same way as are other forms of cancer in the skin and exposed tissue. Furthermore, other conditions and diseases of the skin and exposed tissue are treated in the same way or in ways that cause discomfort to the patient, for example melanoma, genital warts, cervical cancer, HPV (Human Papilloma Virus).

Actinic keratoses lesion is dealt with similarly. Liquid nitrogen is also used to remove the lesion.

These diseases and conditions are usually found in the epidermis (at least for the most part, extending into the dermis and upwards through the Stratum Corneum).

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Hyaluronic acid is a naturally occurring glycosaminoglycan. Its molecular weight may vary from 50,000 daltons upwards, and it forms highly viscous solutions. As regards the actual molecular weight of hyaluronic acid in natural biological contexts, this is still a matter of much uncertainty; when the molecular weight of hyaluronic acid is to be determined, different values are obtained depending on the assay method employed, and on the source, the isolation method etc. The acid occurs in animal tissue, e.g. spinal fluid, ocular fluid, synovial fluid, cockscombs, skin, and also in some streptococci. Various grades of hyaluronic acid have been obtained. A preparation with an allegedly high degree of purity and alleged to be entirely free from side effects, is a non-inflammatory form described in U.S. Patent No.4,141,973; this preparation is said to have a molecular weight exceeding 750,000 dalton, preferably exceeding 1,200,000 dalton and is suggested for therapeutic use in various articular conditions. Applicants believe that hyaluronic acid claimed in this patent is sold under the trade mark Healon.

United States Patent 4,801,619 relates to hyaluronic acid,having a molecular weight of about 3×10^6 dalton or more, administered intra-articularly which is prone to decrease the proteoglycan content of synovial fluid to almost normal levels. According to this patent, this indicates a positive effect on the proteoglycan metabolism of a joint. According to the patent, this is applicable both to inflammatory conditions and to degeneration caused by treatment with symptomatics, such as corticosteroid preparations. It is thus clear that a sufficiently high molecular weight of the hyaluronic acid is alleged to counteract side effects that might be caused by corticosteroids or other symptomatics producing similar effects. When corticosteroids are applied, the amount of hyaluronic acid in the synovial cavity will, according to the patent, increase substantially and, according to the inventors, their hyaluronic acid preparations have a very positive effect on such clinical symptoms as pain, swelling, and lameness.

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The patent states that the objectives of the invention are attained by intra-articular administration (injection) of an effective amount of hyaluronic acid with a mean molecular weight exceeding 3 X 10^6 dalton, preferably exceeding 4 X 10^6 dalton; usually the molecular weight will not exceed 7 X 10^6 dalton. The dosage of hyaluronic acid administered is stated to be preferably within the range of 5mg-80mg. The amount of solution given at each administration is generally less than 60 ml, e.g. less than 20 ml. of an aqueous solution of the acid or its salt. It is convenient to administer the acid dissolved in water (<2% w/w, buffered to physiological pH), for instance in the form of a water-soluble sodium salt. The exact amount will depend on the particular joint to be treated.

The Merck Index Specifies that Hyaluronic Acid has a Molecular Weight within the range pf 50,000 to 8 X 10^6 depen ding on source, methods of preparation, and methods of determination. The Merck Publication teaches hyaluronic acid as a surgical aid (ophthalmological).

United States Patent 4,808,576 purports to teach that hyaluronic acid, an agent well known for reducing the sequelae of trauma in mammalian joint tissue when injected directly into the traumatized joint tissue, will be carried to such traumatized tissue by the mammal's natural processes if applied at a site remote from the traumatized tissue. Thus, hyaluronic acid in any therapeutically acceptable form can, according to the Patent, be administered by the typical remote routes including intravenous, intramuscular, subcutaneous, and topical.

This, the patent alleges, makes the utilization of hyaluronic acid much more convenient and attractive. For instance, the treatment of arthritis in horse or human joints with hyaluronic acid, according to the patent, no longer requires more difficult intra-articular injections.

United States Patent 4,725,585 relates to a method of enhancing or regulating the host defence of a mammal by administering to a mammal a therapeutically effective amount of hyaluronic acid.

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At column 1, lines 43 - 46, the patent provides that the invention was based on the unexpected discovery that administration of hyaluronic acid to mammals results in a considerable increase in the defence.

The hyaluronic acid employed in the patent was Healon (t.m) provided by Pharmacia AB, Uppsala, Sweden (Pharmacia AB is also entitled to the benefit of United States Patent 4,141,973). The patent provides at column 4, line 19 that because a patient's infections had been hard to treat, instead of just hyaluronic acid being administered to the patient to increase the patient's defence, the patient was given hyaluronic acid and an antibiotic. While one reading the patent may conclude that the antibiotic was given in combination with hyaluronic acid, in fact because the hyaluronic acid was administered subcutaneously and because the patient was a heart patient, one skilled in the art would understand that any antibiotic administered, while possibly administered simultaneously with the administration of the hyaluronic acid, was definitely administered separately intravenously (probably) or intramuscularly (less probably). Thus, the hyaluronic acid administered, according to the teachings of this patent, was administered in order to prevent possible development of infections (increase the host's defence) and not for any other reason.

United States Patent 4,636,524 discloses cross-linked gels of hyaluronic acid, alone and mixed with other hydrophilic polymers and containing various substances or covalently bonded low molecular weight substances and processes for preparing them. These products are alleged to be useful in numerous applications including cosmetic formulations and as drug delivery systems.

The patent further states that as hyaluronic acid is known to be a biologically tolerable polymer in the sense that it does not cause any immune or other kind of response when introduced into a human body, the cross-linked hyaluronic acid gels can be used for various medical applications. The cross-linked gels modified with other polymers or low molecular weight substances, it is alleged, can be used as drug delivery devices. For example, the inventors are alleged to have

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found that heparin introduced in a cross-linked hyaluronic acid gel retained its antithrombogenic activity.

The inventors also allege that they have also found that cross-linked gels of hyaluronic acid can slow down the release of a low molecular weight substance dispersed therein but not covalently attached to the gel macromolecular matrix.

Unites States Patent 4,736,024 purports to teach new medicaments for topical use containing:

(i) an active pharmacological substance or a mixture of pharmacological substances, either active or suitable for topical administration and

(ii) a topical vehicle which comprises hyaluronic acid or a molecular fraction of hyaluronic acid or a salt of the same with an alkaline metal, an alkaline earth metal, magnesium, aluminium, ammonium, or a pharmacological substance optionally together with additional conventional excipients for pharmaceutical preparations for topical use.

Applicants are also aware of published Japanese Patent Document 61000017, dated 86/01/06, whose English abstract of disclosure states that the Japanese Patent Document relates to the use of hyaluronic acid or cross-linked hyaluronic acid or their salts as the active ingredient for inhibiting carcinoma metastasis.

According to the purported abstract of the patent, more that 1.0% of hyaluronic acid is dissolved in alkaline aq. soln. and pref. more than 50% of H_2O sol. org. solvent. eq. alcohol, acetone, dioxane, against total soln. is added. Preferably the pH is 12-14. Then a multifunctional epoxy cpd. is added and reacted at 10-60 deg. C, pref. at 20-40- deg. C for 24 hrs. Cross-linking ratio of crosslinked hyaluronic acid or its salt is regulated by changing mol ratio of hyaluronic acid or its salt and multifunctional epoxy cpd.. Pref. hyaluronic acid used has intrinsic viscosity 0.2-30,m.w. 4000-2000000. The hyaluronic acid is allegedly used in several dosage forms. The clinical dose for an adult is alleged to be normally, as hyaluronic acid or cross-linked hyaluronic acid, 25mg-5 g/day (p.o.) and 10 mg-2.5 g/l dose (inj). The abstract alleges that the advantage is that the hyaluronic

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acid has no side effects as may other anti-cancer drugs and has an analgesic and a tissue restoration effect.

European Patent Application 0295092 purports to teach a vehicle together with fragments of hyaluronic acid for delivering of the fragments of hyaluronic acid into the skin to reach the dermal layer of the skin to increase the development of blood vessels for stimulating hair growth or regrowth. The preferred fragments of hyaluronic acid are polysaccharides containing from 7 to 25 monosaccharide units. The patent provides that it is apparent that the larger the fragments of hyaluronic acid, the greater the difficulty there is in delivering the fragments to the dermal layer of the skin, unless there is also present in the composition a means for enhancing the activity of said fragments.

The combination may thus include a means for enhancing the activity of the fragments of hyaluronic acid, especially to improve their penetration through the skin following topical application. Some activity enhancers, it is alleged, also function as vehicles for the fragments of the hyaluronic acid.

Some activity enhancers are also alleged to possess the ability to stimulate or increase hair growth. Minoxidil is asserted among others to be such an activity enhancer. Thus both the fragments of hyaluronic acid and minoxidil are alleged to stimulate hair growth both delivered by a vehicle.

European Patent Application 0179442 asserts that where free radicals are formed in considerable quantities, hyaluronic acid is broken down or degraded before the hyaluronic acid has given the desired effect.

Canadian Letters Patent 1,240,929 teaches the combination of chondroitin sulfate compound and a hyaluronate to protect both human and animal cell layers and tissue subject to exposure to trauma.

European Patent Application 0208623 purports to teach hyaluronic acid as "une augmentation de l'activité de certaines proteases". It also purports to teach the use of hyaluronic acid for treating connective tissue diseases, including malignant tumours and cardiovascular disorders.

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European Patent Application 270317 purports to teach the combination of an antiviral agent lacking inhibitory action and a compound [for example, hyaluronic acid] possessing cell fusion inhibitory activity and/or virus-adsorption inhibitory activity for treating disease carried by a virus.

United States Patent 4,840,941 purports to teach the use of an effective amount of hyaluronic acid as the active agent for the treatment of retroviruses in association with a pharmaceutically acceptable carrier, diluent, or excipient.

United States Patent 4,851,521 and European Patent Application 0265116 both describe hyaluronic acid fractions, the making thereof and cross-linked esters of hyaluronic. United States Patent 4,851,521 describes esters of hyaluronic acid incorporated into pharmaceutical preparations as the active ingredient and as vehicles for ophthamological medicines for topical use (See column 11, lines 35 to 42; and column 12, lines 62 to column 13, line 3) and in suppositories for a systemic effect due to the effect of transcutaneous absorption, such as in suppositories.

The patent provides at column 13, lines 5 to 31:

> "The vehicling action of the hyaluronic esters also applies to associated medicaments of the type mentioned above in which the active substance acts not only topically or by nasal or rectal absorption, for example by nasal sprays or preparations for inhalation for the oral cavity or the pharynx, but also by oral or parenteral route, for example by intramuscular, subcutaneous or intravenous route, as it favors absorption of the drug into the application site. The new medicaments can therefore be applied, apart from in the fields already mentioned, in practically all sectors of medicine, such as internal medicine, for example in pathologies of the cardiovascular system, in infections of the respiratory system, the digestive system, the renal system, in diseases of an endocrinological nature, in

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oncology, in psychiatry etc., and may also be classified therefore from the point of view of their specific action, being perhaps anesthetics, analgesics, anti-inflammatories, wound healers, antimicrobics, adrenergic agonists and antagonists, cytostatics, antirheumatics, antihypertensives, diuretics, sexual hormones, immunostimulants and immunosuppressants, for example, one of the drugs having the activity already described for the therapeutically active alcohols to be used as esterifying component according to the present invention, or for the therapeutically active bases used for the salification of the free carboxylic groups."

There have been extensive studies to determine the defect in immune function that allows a tumour cell to develop. It was postulated initially by Jerne, and subsequently by Burnett, that the immune system's major role was that of immunological surveillance to destroy abnormal cells. The concept of surveillance, while somewhat simplistic, remains an accepted concept for the elaborate mechanism of immune recognition and function that is present in the higher species - mammals.

It has then been postulated that tumours develop because of local or generalized immune suppression. However, as pointed out by Moller, if general immune suppression occurs, it is only certain types of neoplastic disorders that develop, mainly those of the lympho-reticular system. This observation is generally correct and represents a major challenge to the immune surveillance theory unless a specific reason can be shown as to why the individual cancer cell can develop plus individually evade the immune system.

It was demonstrated experimentally in 1974 that defects of macrophage function may exist in neoplastic disease.

The initial experiments found suppressor cells to be part of the immune system; these were either of the T-cell type of the macrophage cell system. There was presence

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demonstrated in neoplasia, chronic bacterial infection, recovery from massive injury and chronic fungal infection.

There has been repeated demonstration in experimental animals that the macrophage cell function is altered in neoplastic disease. The macrophages in the animal's systems appeared "blocked" in their function. Generally when removed from the *in vivo* situation, washed in saline and cultured, they perform normally. This block has been shown to be related to the excessive production of prostaglandin by neoplastic tissue or by the macrophage itself. Similarly, the N.K. cells (which are said to be primitive or immature macrophages and which may be involved in cancer defence) are also blocked.

In the basic research efforts in the latter '70s and the early '80's, there existed considerable confusion as to what role immunotherapy should take in cancer. Activation or "hyping" of macrophages was thought to be important. However, in an examination by Romans and Falk of peritoneal macrophages obtained from patients with neoplastic disease, there was definite evidence that these macrophages were already activated yet were co-existing with cancer cells and not causing their destruction.

It has recently been shown by several independent investigators that the malfunction of macrophages or the putitive block is due to excessive prostaglandin and that this can be altered in tissue culture by corticosteroids, ASA, and the non-steroidal anti-inflammatory drugs, i.e. indomethacin and naproxen (Naprosyn™). Again, it was repeatedly demonstrated that in animal tumours these substances could alter the response to neoplastic cells and that various combinations of these substances employed with immune enhancing agents could produce very credible success in eliminating experimental tumours. Lala and co-workers combined Indomethacin therapy with Interleukin 2 and showed that this could effect a cure with experiment neoplasm.

There were continued problems with the use of any of these agents in the actual human *in vivo* experience. All of the non-steroidal anti-inflammatory agents (NSAID) produced major toxicity in terms of gastro-intestinal, neurological,

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and other areas. Thus, the basis of the present approach is that, under general circumstances, with the use of these agents in human disease in sufficient amounts, the drug will penetrate to any pathological tissue to alter therapeutically local prostaglandin production. While intravenous preparations of Indomethacin (and now of other agents) exist, using these drugs alone produces prohibitive side effects in human subjects. Therefore, only insufficient amounts can be brought into the body to effect more than occasional responses in neoplasm.

However, the majority of the evidence is present to indicate and therefore, it can be postulated that the basis for neoplastic development and how the initial cell "sneaks by" the immune surveillance mechanism relates to its production of prostaglandin. One need postulate only one mutation to alter the amount of prostaglandin synthesis produced by cells when they become "malignant" to establish a mechanism of blocking out the initial cell in any immune reaction, i.e. the macrophage. It therefore became essential to develop a combination of NSAIDs for clinical use to produce a major improvement in response in neoplastic disease and other conditions where excessive prostaglandin synthesis represents the basis of the pathogenesis of this disease state, i.e. arthritis and various others of the so-called connective tissue inflammatory disorders and/or auto-aggressive diseases.

See also:

1. Modulation of Immunity in Cancer Patients by Prostaglandin Antagonists, Immunity to Cancer II, Alan R. Liss, Inc.; and

2. Goodwin, J.S., (1981) Prostaglandin E and Cancer Growth Potential for Immunotherapy with Prostaglandin Synthesis Inhibitors, Augmentive Agents in Cancer Therapy, Raven Press, New York.

United States Patent 4,711,780 teaches a pharmaceutical composition comprising Vitamin C, a zinc salt, and a sulfur amino acid for treating surface epithelium for epithelium regeneration. Hyaluronic acid may be added for

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applications in the reproductive tract to block the passage of toxins into the blood system.

U.S. Patent 4,937,254 (Ethicon) teaches combinations of hyaluronic acid and salts thereof with NSAIDS for the prevention of adhesions after surgery.

Because of the side effects of the use of non-steroidal anti-inflammatory drugs (major toxicity in terms of gastro-intestinal, neurological, and other areas),use thereof should also be restricted (if possible) to the area of use without delivery to other areas which are not in need of treatment. Thus, if useful amounts of the non-steroidal anti-inflammatory drugs or for that matter any drugs could be delivered to a site in need thereof without carriage of substantial amounts away from the site to be treated, thereby accumulating an amount of the drug at the site to be treated for a prolonged period of time, then the use of the drug for example a non-steroidal anti-inflammatory drug at a site may have many other useful applications.

SUMMARY OF THE INVENTION

Applicants have now developed compositions, (combinations and formulations) which are topically applied to the skin and/or exposed tissue of a human and which are quickly transported in dosage amounts percutaneously (intracutaneously) at a site in need of treatment, (site of pathology and/or trauma) best targeting the epidermis and subsequently remaining (accumulating) at the site for a prolonged period of time. The compositions subsequently clear through the lymphatics thereby bringing dosage amounts of the compositions to the lymphatics for the treatment of disease and conditions in the lymphatics.

These compositions, (combinations and formulations) employ, combine, or incorporate (as the case may be) a plurality of effective non-toxic dosage amounts, each dosage amount comprising an effective non-toxic dosage amount of a drug for example a drug which inhibits prostaglandin synthesis for example an NSAID and an effective non-toxic dosage amount of a form of hyaluronic acid (preferably hyaluronic acid or salt thereof) for the transport of the drug to the site of the pathology and/or trauma. Suitable dosage amounts of the

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composition may be removed from a container (for example a tube or jar) and administered (for example, applied).

Thus according to one aspect of the invention these pharmaceutical compositions (combinations and formulations) comprise a plurality of effective non-toxic dosage amounts for administration to the skin and/or exposed tissue of a human in need of treatment, each such dosage amount comprising a therapeutically effective non-toxic (to the patient) dosage amount of a drug to treat a disease and/or condition for example a drug which inhibits prostaglandin synthesis, preferably being a non-steroidal anti-inflammatory drug (NSAID), for example, diclofenac, indomethacin, naproxen, and (+/-) tromethamine salt of ketorolac (sold under the trademark Toradol™) and an effective non-toxic dosage amount (for example in excess of 5 mg per cm^2 (square centimeter) of skin or exposed tissue to which the dosage amount of the composition is to be applied) of hyaluronic acid and/or salts thereof (for example the sodium salt) and/or homologues, analogues, derivatives, complexes, esters, fragments, and/or sub units of hyaluronic acid (preferably hyaluronic acid and/or salts thereof) to transport (to faciliate or cause the transport of) the drug to the site of the pathology and/or trauma of the disease or condition. These compositions may be applied topically to treat diseases and conditions of the skin and/or exposed tissue at the site of the trauma and/or pathology, (for example, basal cell carcinoma, the precancerous, often recurrent, actinic keratoses lesions, fungal lesions, "liver" spots and like lesions (found for the most part in the epidermis), squamous cell tumours, metastatic cancer of the breast to the skin, primary and metastatic melanoma in the skin, malignancies and/or tumours in the skin, genital warts (condyloma acuminata), cervical cancer, and HPV (Human Papilloma Virus) including HPV of the cervix, psoriasis (both plaque-type psoriasis and nail bed psoriasis), corns on the feet and hair loss on the head of pregnant women). The results of the treatment with suitable dosage amounts taken from these compositions (combinations and formulations) have been in some instances quite dramatic - difficult situations have been successfully treated and resolved.

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Furthermore, application of the dosage amounts of the compositions, combinations and formulations are, systemic independent (there is a lack of a blood level of the drug for example NSAID), are quick to penetrate into the skin to the site of the trauma and/or pathology because the effective dosage amount of the form of hyaluronic acid transports (facilitates or causes the transport of) the drug (for example NSAID) particularly to the epidermis where the composition, combination or formulation accumulates and remains for prolonged periods. The compositions subsequently clear through the lymphatics and are available for the treatment of disease and conditions of the lymphatics.

In this regard effective amounts of the form of hyaluronic acid exceeds in the order of about 5 mg per square cm.(cm^2) of the area of for example the skin and/or exposed tissue to which the dosage amounts of the composition is to be applied.

Thus, according to another aspect of the invention, Applicants have provided topically applicable percutaneous (intracutaneous) penetrating (best targeting the epidermis) systemic independent acting (not acting essentially through the blood) pharmaceutical compositions (combinations and formulations) comprising a plurality of dosage amounts each comprising, together with pharmaceutical excipients suitable for topical application, a therapeutically effective (to treat and to assist to resolve diseases and conditions of the skin and exposed tissue (for example basal cell carcinoma, the precancerous, often recurrent, actinic keratoses lesions, fungal lesions, "liver" spots and like lesions (found for the most part in the epidermis), squamous cell tumours, metastatic cancer of the breast to the skin, malignancies and/or tumours in the skin primary and metastatic melanoma in the skin, genital warts (condyloma acuminata), cervical cancer, and HPV (Human Papilloma Virus) including HPV of the cervix, psoriasis (both plaque-type psoriasis and nail bed psoriasis), corns on the feet and hair loss on the head of pregnant women), non-toxic (to the patient) dosage amount of a drug for example which inhibits prostaglandin synthesis, preferably a non-steroidal anti-inflammatory drug (NSAID), for example,

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diclofenac, indomethacin, naproxen, and (+/-) tromethamine salt of ketorolac (sold under the trademark Toradol™) and an effective non-toxic amount of hyaluronic acid and/or salts thereof (for example, the sodium salt) and/or homologues, analogues, derivatives, complexes, esters, fragments, and/or sub-units of hyaluronic acid (preferably hyaluronic acid and salts thereof) to transport (facilitate or cause the transport of) the drug (for example NSAID's) rapidly to the site in the skin (for example epidermis) and/or exposed tissue of the disease or condition into the tissue to remain there for a prolonged period of time to assist to treat and assist to resolve the disease or condition for example by blocking prostaglandin synthesis.

Effective dosage amounts of the form of hyaluronic acid to facilitate or cause the transport of the drug into the skin and/or exposed tissue by the form of hyaluronic acid exceeds about 5 mg. - 10 mg. in the dosage amount administered (applied and rubbed in) for each 1 cm^2 of skin and/or exposed tissue area of the disease or condition (for example basal cell carcinoma) to which the dosage amount is applied. The dosage amount applicable will depend upon the surface area of the skin and/or exposed tissue in which the condition or disease exists. Thus if the disease or condition occupies about .5 cm^2, in excess of about $2^1/_2$ mg of the form of hyaluronic acid would be used (applied and rubbed in). In the same way if the area is 2 cm^2, the amount of the form of hyaluronic acid preferably exceeds about 10-20 mg of the dosage amount of the formulation or composition applied. Preferred forms of the hyaluronic acid (for example hyaluronic acid and the sodium salt thereof) have molecular weights less than about 750,000 daltons (for example about 150,000 to about 225,000 daltons) to transport the medicine in the skin and/or exposed tissue. While higher molecular weights of the hyaluronic acid and forms thereof may be used to penetrate the skin and/or exposed tissue and transport the medicines or drugs, where the molecular weight of the hyaluronic acid chosen for use is very large, it is preferred that the form of hyaluronic acid is autoclaved, to break down the hyaluronic acid to fragments of lesser molecular weight or if feasible

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diluted to permit administration and ensure no coagulation on or in the skin. Where the molecular weight of the form of hyaluronic acid being employed is large, the concentration of the form of the hyaluronic acid in the composition may for example be reduced (for example to less than about 3%) dependent on the molecular weight.

The blockage of prostaglandin synthesis by the transported drug (for example NSAIDS) then unblocks the macrophages and permits the macrophages of the patient proximate the lesion (for example, the basal cell carcinoma) to destroy the lesion or condition. Treatment by dosage amounts of the composition (formulation and/or combination) eliminates the condition without recurrence, even where the lesion has recurred a number of times after unsuccessful treatments according to the prior art.

Other non-steroidal anti-inflammatory drugs (NSAIDS) may be used such as other propionic acid derivatives, Ibuprofen, acetylsalicylic acid, piroxicam and flunixin.

When dosage amounts of such compositions, combinations and formulations are applied to the site of the disease or condition for example the basal cell carcinoma of the patient suffering from the basal cell carcinoma, over a period of time (for example, for a period of 2-4 weeks 3 times daily) the basal cell carcinoma is completely resolved and disappears.

Thus according to another aspect of the invention there is provided a pharmaceutical composition from which dosage amounts may be taken for application to the skin and/or exposed tissue, the pharmaceutical composition comprising in a form for application to a human a plurality of dosage amounts of medicine and/or therapeutic agent to treat a disease or condition in a human and a plurality of dosage amounts of hyaluronic acid and/or salts and/or homologues, analogues, derivatives, complexes, esters, fragments, and/or sub-units of hyaluronic acid such that when dosage amounts of the pharmaceutical composition are taken from the composition, the amount of the medicine and/or therapeutic agent comprises an effective non-toxic dosage amount of the medicine to treat the disease or condition in the skin and/or exposed tissue in a

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human and the amount of the form of hyaluronic acid in the dosage amount is present in an effective amount to transport (facilitate or cause the transport of) the medicine and/or therapeutic agent intradermally (percutaneously, intercutaneously, intracutaneously) into the skin (preferably to the epidermis and dermis) and/or exposed tissue of a human to the site of a pathology and/or trauma. The effective amount of the form of hyaluronic acid has a molecular weight and concentration to transport the medicine (drug) and/or therapeutic agent to the site of trauma and/or pathology in the skin and/or exposed tissue. In this regard the preferred amount of the form of hyaluronic acid in each dosage amount exceeds 5 mg./cm^2 and preferably the molecular weight is less than about 750,000 daltons, (in one embodiment about 150,000 to about 225,000 daltons) in some embodiments with a concentration of between about 1 and 3%, preferably concentrations of between about 2 to about 3% by weight. Where forms of hyaluronic acid are used having greater molecular weights, they are preferably cleaved and/or diluted to smaller concentrations, to facilitate or cause the transport of the medicine and/or therapeutic agent.

According to another aspect of the invention there is provided a pharmaceutical composition (for example a gel or cream) from which dosage amounts may be taken and applied to the skin to treat a disease or condition in humans, for example as discussed above, the pharmaceutical composition comprising:

(1) a medicinal and/or therapeutic agent suitable for treating a disease or condition in the skin and/or exposed tissue in humans, for example a drug which inhibits prostaglandin synthesis (for example an NSAID); and

(2) hyaluronic acid and/or salts thereof and/or homologues, analogues, derivatives, complexes, esters, fragments, and sub-units of hyaluronic acid, in a form suitable for administration to the skin and/or exposed tissue in humans; characterized in that an effective non-toxic dosage amount comprising components (1) and (2) taken and administered from said composition (i) is available in the skin and/or exposed tissue upon administration to treat said

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disease or condition in humans by penetration at the site to be treated to the site of trauma and/or pathology, and (ii) comprises an effective non-toxic dosage amount of component (2) effective to transport (facilitate or cause the transport of) component (1) immediately upon administration percutaneously into the skin (preferably the epidermis) to the site to be treated for example the site of trauma and/or pathology where it remains for a prolonged time, accumulating there and from which it is discharged via the lymphatic system.

Therefore according to another aspect of the invention a pharmaceutical composition is provided comprising:

(1) a medicinal and/or therapeutic agent which for example inhibits prostaglandin synthesis in a therapeutically effective amount to treat a disease or condition of the skin and/or exposed tissue;

and (2) hyaluronic acid and/or salts thereof and/or homologues, analogues, derivatives, complexes, esters, fragments, and subunits of hyaluronic acid,

characterized in that said composition

(a) is in a dosage form (for example a gel or cream) which is suitable for administration to the skin and/or exposed tissue;

and (b) is in such an amount and in such form that (i) component (1) is in an effective dosage amount to treat said disease or condition by penetration at the site of the skin and/or exposed tissue to be treated for example the basal cell carinoma and other lesions; and (ii) component (2) is immediately available to transport (facilitate or cause the transport of) component (1) to the site of trauma and/or pathology to be treated, percutaneously into the skin (or exposed tissue) where the composition resides and accumulates for a prolonged period, and which component (2) is in an effective non-toxic dosage amount to transport (facilitate or cause the transport of) component (1) upon administration, percutaneously into the skin or exposed tissue to the site of the trauma and/or pathology. Preferably the form of hyaluronic acid in the composition comprises hyaluronic acid and/or salts thereof. An effective amount of the form of hyaluronic acid

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exceeds about 5-10 mg per square centimeter (cm^2) of skin and/or exposed tissue to which it is to be applied.

According to another aspect of the invention there is provided the use of:

(1) a medicinal and/or therapeutic agent for example which inhibits prostaglandin synthesis,

and (2) hyaluronic acid and/or salts thereof and/or homologues, analogues, derivatives, complexes, esters, fragments, and subunits of hyaluronic acid,

in the manufacture of a pharmaceutical composition for treating a disease or a condition (for example those discussed above) of the skin and/or exposed tissue in a therapy wherein dosage amounts taken from the composition each comprise:

(1) a therapeutically effective amount of said medicinal and/or therapeutic agent and

(2) a therapeutically effective amount of the hyaluronic acid and/or salts thereof and/or homologues, analogues, derivatives, complexes, esters, fragments, and sub-units of hyaluronic acid, the pharmaceutical composition being characterized in that for each dosage amount taken from the pharmaceutical composition, the amount of component (2) is immediately available to transport component (1) percutaneously to the site of trauma and/or pathology for example into the epidermis where the composition accumulates and remains for a prolonged period, at the site of the skin or exposed tissue to be treated, and component (2) is in an effective non-toxic amount to transport (facilitate or cause the transport of) component (1) into the skin or exposed tissue (for example into the epidermis). Preferably component (2) is hyaluronic acid and/or salts thereof and preferably the dosage amount of component (2) in the amount of the composition taken from the composition (to be taken from the composition) and applied to the skin or exposed tissue is a dose amount greater than about 5-10 mg per cm^2 of skin and/or exposed tissue to which the dosage amount is to be applied.

The pharmaceutical composition will normally include pharmaceutically compatible excipients to provide a form for ease of administration to the skin and/or exposed tissue for

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transport into the epidermis. For example a suitable dosage amount of a gel may be squeezed from a tube as a ribbon of gel "X" cm long (which dosage amount (in the form of the ribbon "X" cm long) contains the effective non-toxic dosage amounts of the drug and form of hyaluronic acid. Or a dosage amount of cream packaged in a jar may be scooped from the jar by a measuring device or by "two fingers" in a suitable amount (for example in a spoon containing a premeasured volume or an amount about half the "length of the fingers"). Each of the dosage amounts selected comprises the effective amounts of drug (for example NSAID) and effective amount of the form of hyaluronic acid (for example hyaluronic acid and/or salts thereof). In this way the patient may "squeeze" or "scoop" or "what have you" the appropriate dosage amount and apply (rub in) the dosage amount onto the skin and/or exposed tissue for transport into the epidermis.

Thus, according to another aspect of the invention, a method of treating a disease and/or condition of the skin or exposed tissue, for example basal cell carcinoma, the precancerous, often recurrent, actinic keratoses lesions, fungal lesions, "liver" spots and like lesions (found for the most part in the epidermis), squamous cell tumours, metastatic cancer of the breast to the skin, primary and metastatic melanoma in the skin, malignancies and/or tumours in the skin, genital warts (condyloma acuminata), cervical cancer, HPV (Human Papilloma Virus) including HPV of the cervix, psoriasis (both plaque-type psoriasis and nail bed psoriasis), corns on the feet and hair loss on the head of pregnant women, in a human is provided comprising administering topically to human skin and/or exposed tissue an effective non-toxic dosage amount of a composition comprising, together with pharmaceutical excipients suitable for topical application to the skin and/or exposed tissue, for example in the form of a gel or cream (to give the composition definition and form so that specific dosage amounts are easily selected or taken for administration (for example squeezed from a tube or scooped from a jar and rubbed into the skin or exposed tissue), a therapeutically effective (to treat and to assist to resolve the disease or condition for example basal cell carcinoma or

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other lesion), non-toxic (to the patient) dosage amount of a drug for example which inhibits prostaglandin synthesis, for example a non-steroidal anti-inflammatory drug (NSAID), for example, diclofenac, indomethacin, naproxen, and (+/-) tromethamine salt of ketorolac (sold under the trademark Toradol™) and an effective non-toxic dosage amount of hyaluronic acid and/or salts thereof (for example, the sodium salt) and/or homologues, analogues, derivatives, complexes, esters, fragments, and/or sub-units of hyaluronic acid (preferably hyaluronic acid and salts thereof) to transport (facilitate or cause the transport of) the drug (for example NSAID) into the skin or exposed tissue to the site of the disease or condition to be treated percutaneously, (to the site of trauma and/or pathology), for example into the epidermis, where the form of hyaluronic acid and medicine accumulates and remains for a prolonged period of time thereby for example blocking prostaglandin synthesis in the skin or exposed tissue. The form of hyaluronic acid is then cleared through the lymphatics (lymphatics system).

Thus, according to another aspect of the invention, the treatment may employ the use of the composition, formulation or combination for the treatment of the diseases and conditions aforesaid as for example by applying dosage amounts of the composition, formulation or combination a number of times daily (for example, 3 times daily) for a period of time, for example, 2-4 weeks to clear the disease, lesion or condition. Each dosage amount applied will depend upon the size of the lesion or condition on the skin or exposed tissue. For example, a suitable dosage amount may include 5-10 mg. of the form of hyaluronic acid per 1 cm^2 skin area or exposed tissue area.

One such formulation may comprise 3% (by weight) diclofenac in a $2\frac{1}{2}$% (by weight) hyaluronic acid (sodium hyaluronate - molecular weight 661,600) gel formulation, with the excipients being glycerine (5%), benzyl alcohol (3%) (acting in part as a solubilizer and preservative), and sterile water (the balance) in a 50 gm. tube of the composition (a plurality of dosage amounts) whose tube O.D. (outer diameter) of the opening through which the gel

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formulation is discharged from the tube is 8 mm and whose I.D. (inner diameter) of the opening is 4 mm. Therefore a ribbon 2-3 cm in length, squeezed from a tube gives about 5 mg-$7^1/_2$ mg of hyaluronic acid for application to a skin or exposed tissue surface area of $1-1^1/_2 cm^2$ with an effective dosage amount of diclofenac. While greater amounts squeezed from the tube, may be applied, the application of substantial excessive dosage amounts to the skin and/or exposed tissue may saturate the skin or exposed tissue and thus the epidermis. (There is therefore no more room for the composition to pass between the cells and therefore further applications at that time will not provide additional benefit). Where pain relief is also required additional dosage amounts, for example in excess of about 10 mg. of the hyaluronic acid taken from the same pharmaceutical composition applied per/cm^2 of surface area of the skin or exposed tissue may be required to be applied.

Another formulation may comprise 3% (by weight) diclofenac in a $2^1/_2$% (by weight) hyaluronic acid (sodium hyaluronate - molecular weight 679,000) gel formulation (also in a tube) with excipients being benzyl alcohol (1%) (a preservative), methoxypolyethylene glycol 350 (20% by weight) (a solubilizer), and sterile water (the balance).

While the above compositions, combinations and formulations are proposed, provided there is sufficient amounts of the form of the hyaluronic acid (for example, sodium hyaluronate) in the dosage amounts applied to the skin and/or exposed tissue to facilitate or cause the percutaneous (intracutaneous) transport of the drug for example which inhibits prostaglandin synthesis, preferably an NSAID (for example, diclofenac) to block prostaglandin synthesis, then the formulations may be of any suitable form, for example, a 1% lotion of hyaluronic acid with NSAID, or a cream or gel or any other suitable form.

Therefore according to another aspect of the invention, there is provided containers (for example tubes and jars) containing compositions comprising a plurality of dosage amounts of the drug and form of hyaluronic acid, each dosage amount comprising an effective non-toxic dosage amount of the drug and an effective non-toxic dosage amount of the form of

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hyaluronic acid (preferably sodium hyaluronate having molecular weight less than about 750,000 daltons) to transport the drug into the skin and/or exposed tissue. In some embodiments, means are provided to assist the removal from the container of an effective dosage amount of the composition in the container for use to apply to the skin or exposed tissue at the site of trauma and/or pathology to treat the disease and/or condition (for example mouth opening of a tube to control the amount discharged from the tube).

Furthermore, because there is little concern with respect to the toxicity or adverse effects of the use of, for example, the NSAIDs with the hyaluronic acid in the compositions of this invention the NSAID may be combined as needed (after solubilizing (if required) of the NSAID in a suitable solubilizer) with the form of the hyaluronic acid.

Therefore according to another aspect of the invention, percutaneous (intercutanous) delivery of a therapeutically effective dosage amount of a drug (in a composition, combination or formulation) and which drug for example inhibits prostaglandin synthesis, preferably being a non-steroidal drug (NSAID) is provided. In this regard the drug is transported to the site of, on, or in the skin and/or exposed tissue of trauma and/or pathology to treat the disease or condition for example the basal cell carcinoma or actinic keratoses lesion in a mammal (human). The delivery may comprise topically administering (to the skin or exposed tissue site of for example the basal cell carcinoma or other lesion) a therapeutically effective non-toxic (to the patient) dosage amount of a composition comprising a drug for example which inhibits prostaglandin synthesis, preferably an NSAID (non-steroidal anti-inflammatory drug), for example, diclofenac, indomethacin, naproxen, and (+/-) tromethamine salt of ketorolac (sold under the trademark Toradol™), and an effective non-toxic amount of hyaluronic acid and/or salts thereof and/or homologues, analogues, derivatives, complexes, esters, fragments, and sub-units of hyaluronic acid, preferably hyaluronic acid and salts thereof, sufficient to transport, (facilitate or cause the transport of), the drug for example NSAID percutaneously (to for example the

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epidermis) to the site of the trauma and/or pathology in for example the epidermis, for example the basal cell carcinoma (or other lesion), to be treated for example to block the synthesis of prostaglandins.

Delivery may be also accomplished by the same amount of the form of hyaluronic acid, of other drugs percutaneously (intercutaneously) to the skin and exposed tissue by application and rubbing in of an effective non-toxic dosage amount of the formulation or composition comprising an effective non-toxic dosage amount of the drug and an effective non-toxic dosage amount of the form of hyaluronic acid for the transport of the drug percutaneously into the skin or exposed tissue to the epidermis where the dosage amount of the composition is accumulated and remains for a prolonged period of time before the form of hyaluronic acid is cleared through the lymphatics. In this regard the drug may be novantrone (an anti-cancer drug) for administration to a tumour or malignancy in the skin. The novantrone may comprise 10 mg in the dosage amount of the composition and the form of hyaluronic acid may be in excess of about 5 mg of sodium hyaluronic per cm^2 of the skin or exposed tissue (about 2.5% of the composition) for the percutaneous transport of the novantrone.

Thus, according to another aspect of the invention, use of a composition, combination or formulation is provided to treat a disease or condition for example basal cell carcinoma (or other lesion), by the application of the composition, combination or formulation, the amount of the composition, combination and formulation administered comprising together with pharmaceutical excipients suitable for topical application, a therapeutically effective (to treat and assist to resolve a disease or condition for example, basal cell carcinoma), non-toxic (to the patient) amount of a drug for example which inhibits prostaglandin synthesis preferably a non-steroidal anti-inflammatory drug (NSAID), for example, diclofenac, indomethacin, naproxen, and (+/-) tromethamine salt of ketorolac (sold under the trademark Toradol™) administered together with, or carried in, an effective dosage amount of hyaluronic acid and/or salts thereof (for example, the sodium salt) and/or homologues,

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analogues, derivatives, complexes, esters, fragments, and/or sub-units of hyaluronic acid (preferably hyaluronic acid and salts thereof) effective to transport the drug for example the NSAID (to facilitate or cause the transport of the drug for example NSAID) percutaneously into the skin especially the epidermis at the site of the disease or condition for example basal cell carcinoma (or other lesion) to be treated, thereby, if an NSAID, blocking prostaglandin synthesis to enable the macrophages (and N.K. cells) to resolve the disease or condition for example basal cell carcinoma or other lesion.

Applicants postulate that the hyaluronic acid and/or salts thereof and/or the homologues, analogues, derivatives, complexes, esters, fragments, and/or sub units of hyaluronic acid facilitate or cause the transport of the drug for example which blocks prostaglandin synthesis (preferably an NSAID) to the site of prostaglandin synthesis to block prostaglandin synthesis.

Applicants' compositions and dosage amounts of their compositions and the use of their compositions and dosage amounts of their compositions, at the same time, abate pain that the patient is experiencing at the paccinian nerve bundles (superficial nerve bundles) at the site of the trauma and/or pathology on/in the exposed tissue and/or skin.

Thus, according to another aspect of the invention, a method of abating pain in the skin and/or exposed tissue for example suffering a disease or condition (for example those discussed above), and a composition from which dosage amounts may be taken and applied (rubbed in) which is useful for abating such pain are provided, the method comprising administering (rubbing on) an effective dosage amount of the composition to the skin and/or exposed tissue, and the composition comprises a plurality of dosage amounts, each comprising an effective non-toxic dosage amount of an NSAID and an effective non-toxic dosage amount of the hyaluronic acid and/or salts thereof and/or homologues, analogues, derivatives, complexes, esters, fragments, and/or subunits of hyaluronic acid (preferably hyaluronic acid and salts thereof), for example amounts exceeding 10-20 mg. per square cm (cm^2) of skin or exposed tissue to which it is applied, for

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percutaneous transport of the NSAID by the form of hyaluronic acid into the epidermis proximate the paccinian nerve bundles (superficial nerve bundles at the end of the nerves) to abate pain. Thus, according to another aspect of the invention, compositions are provided for use to relieve pain from which dosage amounts of the composition comprising dosage amounts of the NSAID and form of hyaluronic acid are taken.

By way of example and to illustrate the facilitation of the delivery or transport of a chemical to a site in a human, when ethyl alcohol is injected directly into a tumour and sonographic (ultrasound) assessment is made, it is not dispersed throughout the tumour. When the ethyl alcohol to be administered into a tumour is carried by hyaluronic acid and/or salts thereof, sonographic assessment of the tumour demonstrates the dispersion of the ethyl alcohol throughout the tumour.

While Applicants postulate that the hyaluronic acid facilitate or causes the transport and delivery, Applicants' invention may be used as described irrespective of the actual method of operation of the hyaluronic acid and/or salts thereof and/or the homologues, analogues, derivatives, complexes, esters, fragments and sub-units of hyaluronic acid.

The combination of hyaluronic acid and salts thereof and other forms with drugs for example that inhibit prostaglandin synthesis, for example NSAIDs, alters their distribution and performance in the skin and/or exposed tissue particularly the epidermis (the combinations and formulations being systemic independent), and produces an unusual targeting for underperfused skin and/or pathological tissue in the skin (site of trauma and/or pathology). The application may be made as required with the amount depending upon the condition of the skin or exposed tissue.

As a major amount of soluble indomethacin may be incorporated into the formulation, or composition, the indomethacin may be solubilized using n-methyl glucamine at a dilution of 5mg/ml of n-methyl glucamine (NMG). This substance is then passed through a 22 micron Milipore filter to produce sterility. This material is non-toxic at 16 fold the therapeutic dose in animals (with hyaluronic acid) and for

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this reason was considered appropriate to be used in human conditions. Thus, Indocid™ solubilized in NMG may be administered with hyaluronic acid topically for percutaneous penetration at, for example, varying doses. The solution of indomethacin and NMG may be mixed with, for example, "LifeCore™" hyaluronic acid in dosage amounts discussed above. This produces an appropriate mixture and can be administered safely.

When the NSAID, for example indomethacin (dissolved in n-methyl glucamine) or other NSAID, is applied topically in an effective dosage amount from a composition or formulation also including the effective dosage amount of the form of hyaluronic acid, no major toxic side effects occur, such as gastro-intestinal distress, neurological abnormalities, depression, etc., even at elevated amounts of indomethacin (if necessary). (This may be in part because of the clearing of the hyaluronic acid through the lymphatic system from the site). In addition, the responses that have been observed are dramatic when the drug for example NSAID (for example diclofenac) is combined with hyaluronic acid, demonstrating clearly that the combination is now "targeting" to the site of pathology or trauma, or pathological tissue. Furthermore, patients using the formulations and combinations of drug (for example NSAID) - hyaluronic acid (sodium hyaluronate) (for example, diclofenac or indomethacin and hyaluronic acid), experience dramatic relief of pain immediately.

Thus, Applicants believe that the use of the NSAID, for example with hyaluronic acid (sodium hyaluronate), deblocks the macrophages (and N.K. cells (Natural Killer Cells) thought to be immature macrophages) by preventing enzymatic production of prostaglandin which blocks macrophage (and N.K. cell) functioning. The hyaluronic acid (and salt and other forms) not only enhances the activity of the drug (NSAID) but also reduces any side effects and toxicity that is associated with the use of the prostaglandin synthesis inhibitors. When effective dosage amounts of compositions, formulations and combinations containing effective dosage amounts of the drugs for example, (NSAIDs (for example, diclofenac)) and effective dosage amounts of, for example,

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hyaluronic acid or the sodium salt thereof, are applied to for example the tumour lesion (for example basal cell carcinoma) or other condition (for example, actinic keratoses lesion) for a period of time (for example, 3 times daily for 2-4 weeks), the carcinoma and lesions, as the case may be, disappear.

Applicants also postulate that when the combination or formulation is applied to the disease or condition (for example, basal cell carcinoma or actinic keratoses), the hyaluronic acid passes between the cells (in the stratum corneum and epidermis to the dermis depending on amounts) to the areas of trauma and/or pathology deficient in hyaluronic acid (or forms thereof), transporting, taking, drawing, carrying or pulling the NSAID with it to the sites of prostaglandin synthesis, penetrating to inhibit prostaglandin synthesis until the space between the cells is saturated. The NSAID now being proximate the Paccinian nerve bundle (superficial nerve bundles at the end of the nerves) gives pain relief. The macrophages (which had been previously blocked) are unblocked and act to destroy the disease or condition for example basal cell carcinoma, actinic keratoses lesion, or other disease or lesion. Furthermore, the effective non-toxic dosage amount of the composition, combination or formulation, comprising the effective dosage amount of the form of hyaluronic acid and the effective dosage amount of NSAID passing through the stratum corneum to the epidermis and to the dermis (if a sufficient amount of the form of hyaluronic acid is present), passes into the skin, accumulating and staying longer in the skin at the site of the trauma and/or pathology. Therefore, after having had an immediate effect at the site of trauma and/or pathology (for example, relieving pain and acting on the basal cell carcinoma, actinic keratoses and other disease, condition or lesion), the NSAID-hyaluronic acid combination continues to accumulate at the site in need of treatment and thereafter clears through the lymphatic system.

Thus according to another aspect of Applicant's invention, Applicants' compositions, formulations and combinations quickly penetrate on application through the stratum corneum into the epidermis (to the dermis) by the form

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of hyaluronic acid transporting the NSAID, to the site of trauma and/or pathology where the amounts applied accumulate and remain for a prolonged time for treatment.

Fifteen (15) minutes after application of one of Applicants' formulations, about three times the amount of Applicants' formulation has penetrated into the skin (particularly the epidermis) than formulations and combinations not containing hyaluronic acid or effective dosage amounts of hyaluronic acid, but containing the same drug. Furthermore, the drug and hyaluronic acid accumulate and remain at the site in need of treatment for a longer period of time.

Thus according to another aspect of the invention, non-toxic effective dosage amounts of forms of hyaluronic acid (preferably sodium hyaluronate) and effective non-toxic dosage amounts of a drug may be administered in compositions to sites of trauma or pathology, on/in the skin and/or exposed tissue (for example the epidermis) by the application of the effective non-toxic dosage amount of the composition comprising an effective non-toxic dosage amount of a drug (for example an NSAID) and an effective non-toxic dosage amount of a form of hyaluronic acid (for example sodium hyaluronate) to the skin or exposed tissue whereby the forms hyaluronic acid transport the drug percutaneously to the site of trauma and/or pathology where the composition accumulates and remains for a prolonged period of time thereby retaining the drug at the site of trauma and/or pathology (for example the epidermis) for the treatment of the condition or disease and the reduction of pain.

Thus according to another aspect of the invention, Applicants have provided compositions (formulations and combinations) (including pharmaceutical excipients suitable for topical application) from which effective non-toxic (to the patient) dosage amounts of a drug (for example an NSAID) to treat and to assist to resolve diseases and conditions of the skin and/or exposed tissue (for example basal cell carcinoma, the precancerous, often recurrent, actinic keratoses lesions, fungal lesions, "liver" spots and like lesions (found for the most part in the epidermis), squamous

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cell tumours, metastatic cancer of the breast to the skin, primary and metastatic melanoma in the skin, malignancies and/or tumours of the skin, gential warts, cervical cancer, and HPV (Human Papilloma Virus) including HPV of the cervix, psoriasis (both plaque-type psoriasis and nail bed psoriasis), corns on the feet and hair loss on the head of pregnant women), and effective non-toxic dosage amounts of hyaluronic acid and/or salts thereof (for example, the sodium salt) and/or homologues, analogues, derivatives, complexes, esters, fragments, and/or sub-units of hyaluronic acid (preferably hyaluronic acid and salts thereof) sufficient to transport (to facilitate or cause the transport of) the drug, for example NSAID, are taken for application, to a site in the skin (for example epidermis) or exposed tissue having a disease or condition for percutaneous transport into the skin and/or exposed tissue to accumulate and remain there for a prolonged period of time to for example block prostaglandin synthesis. Thus an effective dosage amount of the composition or formulation or combination penetrates quickly into the skin, for example by the hyaluronic acid transporting the NSAID or causing the NSAID to be transported for example to the epidermis of the skin, accumulates there and remains there for a prolonged period of time, thereby accumulating the drug and forms of hyaluronic acid in the skin (particularly the epidermis).

Thus according to another aspect of the invention, a method of accumulating a drug and a form of hyaluronic acid in skin and/or exposed tissue is provided comprising topically administering a therapeutically effective non-toxic dosage amount of a composition comprising pharmaceutical excipients suitable for topical applications, an effective non-toxic (to the patient) dosage amount of a drug for example which inhibits prostaglandin synthesis, preferably a non-steroidal anti-inflammatory drug (NSAID), for example, diclofenac, indomethacin, naproxen, and (+/-) tromethamine salt of ketorolac (sold under the trademark Toradol™) (to treat and to assist to resolve the disease and conditions of the skin and exposed tissue (for example basal cell carcinoma, the precancerous, often recurrent, actinic keratoses lesions,

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fungal lesions, "liver" spots and like lesions (found for the most part in the epidermis), squamous cell tumours, metastatic cancer of the breast to the skin, malignancies and/or tumours of the skin, primary and metastatic melanoma in the skin, genital warts cervical cancer, and HPV (Human Papilloma Virus) including HPV of the cervix, psoriasis (both plaque-type psoriasis and nail bed psoriasis), corns on the feet and hair loss on the head of pregnant women), and an effective non-toxic dosage amount of hyaluronic acid and/or salts thereof (for example, the sodium salt) and/or homologues, analogues, derivatives, complexes, esters, fragments, and/or sub-units of hyaluronic acid (preferably hyaluronic acid and salts thereof) effective to transport (to facilitate or cause the transport of) the drug (for example NSAID) percutaneously to the site in the skin (for example epidermis) or exposed tissue of the disease or condition to accumulate and remain there for a prolonged period of time for example to block prostaglandin synthesis.

According to another aspect of the invention, a method of quickly delivering a drug to the skin or exposed tissue, particularly the epidermis, and maintaining the drug therein for a prolonged period of time is provided, the method comprising topically administering (for example rubbing in) an effective non-toxic dosage amount of a composition comprising pharmaceutical excipients suitable for topical application, a therapeutically effective (to treat and assist to resolve the disease and/or condition of the skin and exposed tissue (for example basal cell carcinoma, the precancerous, often recurrent, actinic keratoses lesions, fungal lesions, "liver" spots and like lesions (found for the most part in the epidermis), squamous cell tumours, metastatic cancer of the breast to the skin, primary and metastatic melanoma in the skin, malignancies and/or tumours of the skin, genital warts, cervical cancer, and HPV (Human Papilloma Virus) including HPV of the cervix, psoriasis (both plaque-type psoriasis and nail bed psoriasis), corns on the feet and hair loss on the head of pregnant women)), non-toxic (to the patient) dosage amount of a drug for example which inhibits prostaglandin synthesis, preferably a non-steroidal anti-

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inflammatory drug (NSAID), for example, diclofenac, indomethacin, naproxen, and (+/-) tromethamine salt of ketorolac (sold under the trademark Toradol™) and an effective non-toxic dosage amount of hyaluronic acid and/or salts thereof (for example, the sodium salt) and/or homologues, analogues, derivatives, complexes, esters, fragments, and/or sub-units of hyaluronic acid (preferably hyaluronic acid and salts thereof) sufficient to transport (to facilitate or cause the transport of) the drug for example the NSAID percutaneously to the site of the trauma and/or pathology in the skin (for example epidermis) or exposed tissue, for remaining there for a prolonged period of time (for example in the epidermis and dermis) to for example block prostaglandin synthesis. Suitable amounts of the form of hyaluronic acid may comprise in excess of 5 mg. per cm^2 in a form which transports the drug (for example molecular weights of the form of hyaluronic acid being less than about 750,000 Daltons or if at substantially greater molecular weights, diluted (to reduce) the concentration or autoclaved or cleaved if required to reduce the size of the molecules.

According to another aspect of the invention, a method of controlling the unloading of a drug from the skin or exposed tissue into the lymphatic system comprises delivering (transporting) an amount of drug into the skin or exposed tissue by an effective non-toxic dosage amount of a form of hyaluronic acid and/or salts thereof and/or homologues, analogues, derivatives, complexes, esters, fragments, and/or sub-units of hyaluronic acid to the skin (epidermis) or exposed tissue to control the unloading of the drug into the lymphatic system (for example by the application of greater than 5 mg./cm^2) of the form of hyaluronic acid.

Thus according to another aspect of the invention a composition is provided which when administered to a human by preferably administration to the skin and/or exposed tissue of a human, unloads its contents into the lymphatic system, the composition comprising an effective non-toxic dosage amount of a drug (for example an NSAID or an anti-cancer drug (Novantrone) and an effective non-toxic amount of hyaluronic acid and/or salts thereof and/or homologues, analogues,

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derivatives, complexes, esters, fragments and/or sub-units of hyaluronic acid (for example at least about 5-10 mg/cm^2 of skin or exposed tissue). Thus the composition is made up of a plurality of such dosage forms (for example a cream or lotion or gel).

Thus according to another aspect of the invention, a new composition for treating diseases via the lymphatic system is provided comprising a plurality of effective non-toxic dosage amounts of the composition, each dosage amount comprising hyaluronic acid and/or salts thereof and/or homologues, analogues, derivatives, complexes, esters, fragments and/or sub-units of hyaluronic acid for passing into the lymphatic system and a therapeutic effective amount of medicine for treatment of a disease (which disease may be in the lymphatic system).

According to another aspect of the invention, the composition may be for application to the skin or exposed tissue.

According to another aspect of the invention, a composition is provided from which effective dosage amounts may be taken and administered, each effective dosage amount of the composition comprising an effective non-toxic dosage amount of hyaluronic acid and/or salts thereof and/or homologues, analogues, derivatives, complexes, esters, fragments and/or sub-units for transporting a therapeutically effective non-toxic dosage amount of a medicine and/or therapeutic agent (for example an NSAID) in the composition into the skin and/or exposed tissue when applied thereto to an area of pathology and/or trauma then into the lymphatic system, the dosage amount being essentially systemic independent such that substantial amounts do not enter the blood system prior to clearing (passing) into the lymphatic system. Preferably the amount of the form of hyaluronic acid in each dosage amount administered is greater than about 5-10 mg./cm^2 and the molecular weight is less than about 750,000 daltons.

We have compared the penetration and retention of one of our combinations (formulations) with a control and Voltarol Emulgel in the skin as follows:

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(A) OUR FORMULATION

1% DICLOFENAC IN 3.0% HA GEL 50g/tube

EPDICLO1

LOT XPB 044 Quantity 1500ml

FORMULA	Supplier	Lot	Amount	Percent
Sterile Water	Baxter	AW45F1	1397ml	--
Glycerin	Life	1043	45g(36ml)	3%
Benzyl Alcohol	Caledon	02517	22.5g(22ml)	1.5%
Liquid Wax DICDD	Brooks	191-175	45g	3%
Diclofenac Sodium	Prosintex	9113003	15g	1%
Sodium Hyaluronate	Skymart	HG-1103	45g	3%
Mol. Wt. 661,600				

PROCEDURE

- Set up stirring apparatus using a 3 liter stainless steel beaker
- Add Water, Glycerin, Benzyl Alcohol and Liquid Wax DICDD, stir and mix for 10 minutes
- Add Diclofenac Sodium and stir for 30 minutes to dissolve
- Add Sodium Hyaluronate and stir for 90 minutes

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FILLED

In a 50 ml aluminum collapsible tube,
inside of tube lacquered with a phanolic resin, outside of tube white regular enamel coating;
9 mm white polypropylene screw on cap with pierce tip

	Gels	Batch No.s
(B)	Voltarol Emulgel	060400 10 93
(C)	1% Diclofenac Gel	XPB049 (Control)

(C) CONTROL

1% DICLOFENAC IN CARAPOL GEL, 50g Jar

LOT XPB 049 Quantity 100ml

FORMULA	Supplier	Lot	Amount	Percent
Sterile Water	Baxter	AW45N5	93ml	--
Glycerin	BDH	2579	3g	3%
Benzyl Alcohol	BDH	23797	1.5g	1.5%
Liquid Wax DICDD	Brooks	L-1424	3g	3%
Diclofenac Sodium	Prosintex	9113003	1g	1%
Carbopol 934	A&C Chemicals	910304	1g	1%

PROCEDURE

- Set up stirring apparatus using a 400ml stainless steel beaker
- Add Water, Glycerin, Benzyl Alcohol, Liquid Wax DICDD, and stir to mix thoroughly for 10 minutes
- Add Diclofenac Sodium and stir for 20 minutes to dissolve
- Very slowly add Carbopol 934, avoid getting lumps

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Samples

Cell	Sample	Quantity of gel applied (mg)
A	060400 10 93	192
B	060400 10 93	192
C	EPDICLO1*	192
D	EPDICLO1*	192
E	XPB049	192
F	XPB049	192

* - Our Formulation

Skin Type

One piece of skin (Female, 37 years, smoker, breast skin) was used for one sample from each batch. A second piece of skin (no further details available) was used for the second sample from each batch. The skin was stored deep frozen (<-20°C) until thawed for this experiment. Full thickness skin was used for this experiment.

Experimental Conditions

Skin permeation cells were prepared containing an exposed skin surface area of 9.6 cm^2 and a constantly stirred receptor fluid beneath the skin consisting of 135 ml of ethanol:phosphate buffered saline (25:75 v/v).

Each cell was allowed to equilibrate for 1 hour at 37°C after which the gel was spread evenly over the skin surface at a concentration of 20 mg/cm^2). See table above. The cell was then maintained at 37°C with an air temperature above the skin of 35°C.

24 hours after application of the gel the experiment was stopped and a portion of the receptor fluid removed. The skin was removed from the cell and any gel remaining on the surface carefully wiped off with dry paper towel followed by paper towel moistened with water. The skin was cut with a scalpel to obtain thin top and thicker lower sections of skin.

This was done in order to obtain layers of skin which approximated the epidermal and dermal layers. Each skin section was weighed and the residual diclofenac extracted with 10ml of fresh receptor fluid using an ultra turrax

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homogeniser. The homogenates were centrifuged and a portion of the resultant supernatant solutions removed.

The receptor fluid and skin extracts from each cell were assayed for diclofenac content by using a validated reverse phase high performance liquid chromatography (HPLC) method.

Results

Distribution of Diclofenac 24 hours after application of Diclofenac Gel

Sample	Receptor	Top Skin portion			Bottom skin portion		
	µg	Skin Weight (g)	µg	µg/g	Skin Weight	µg	µg/g
(Voltarol Emugel)							
060400 10 93	447	0.1363	101	742	1.2449	217	174
060400 10 93	764	0.2445	141	577	1.2351	202	164
Mean	606			660			169
(Our Formulation)							
EPDICLO1	247	0.1535	133	867	1.4663	148	101
EPDICLO1	292	0.1647	145	879	1.0022	86	86
Mean	269			873			93
(Control)							
XPB049	184	0.1275	35	272	1.1324	58	51
XPB049	147	0.2068	82	396	1.0893	68	63
Mean	165			334			57

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Thus having regard to the above and Figures 1', 2' and 3', it is clear that the sodium hyaluronate takes the diclofenac into the skin to the epidermis level (See Figure 1') more rapidly than the Voltarol Emugel or non-hyaluronic acid diclofenac containing control formulation, accumulates it there and retains it there longer. The other formulations permit the NSAID, diclofenac, to pass through the bottom skin portion (dermis) quicker, thereby clearing it from the epidermis and dermis, quicker. Furthermore, more of Applicants' formulation is in the epidermis and in the dermis even after 12 hours. With respect to Figure 1', the top of the graph should have the following heading "DICLOFENAC TOP SKIN PORTION", the left side of the graph should have the following side heading "DICLOFENAC (MICROGRAMS) (THOUSANDS)" and the bottom of the graph should have the following bottom heading "ELAPSED TIME (HOURS) ◻ 0604001093 + EPDICLO1 ◊ XP8049". With respect to Figure 2', the top of the graph should have the following heading "DICLOFENAC BOTTOM SKIN PORTION", the left side of the graph should have the following side heading "DICLOFENAC (MICROGRAMS)" and the bottom of the graph should have the following bottom heading "ELAPSED TIME (HOURS) ◻ 0604001093 + EPDICLO1 ◊ XP8049". With respect to Figure 3', the top of the graph should have the following heading "DICLOFENAC RECEPTOR SOLUTION", the left side of the graph should have the following side heading "DICLOFENAC (MICROGRAMS)" and the bottom of the graph should have the following bottom heading "ELAPSED TIME (HOURS) ◻ 0604001093 + EPDICLO1 ◊ XP8049".

It is also clear that Applicants' formulations clear into the lymphatic system not through the blood system. Yet the prior art topical formulations have always tried "to drive" the formulations through the skin into the blood for treatment of the disease or condition in the area (i.e. systemic action).

Thus, our composition, formulation and combination, (and dosage amounts thereof) penetrate quickly and rapidly at the site of treatment through the upper skin into the epidermis, where the paccinian bundles are located and the NSAID and the form of hyaluronic acid are accumulated and are

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retained longer, where needed (for example for the treatment of basal cell carcinoma).

Further, the NSAIDs are retained in the area to be treated with the form of hyaluronic acid. In doing so, they preclude prostaglandin synthesis , in effect, deactivating the synthesis or inhibiting the synthesis, of prostaglandins, permitting the macrophages' scavenger cell activity to eliminate the tumour and lesion. Additionally, a rapid onset of pain relief (analgesic effect) is provided (depending on the amount of NSAID and form of hyaluronic acid) usually where in excess of about 10 mg of the form of hyaluronic acid (preferably hyaluronic acid and salts thereof) is administered per cm^2 of surface area comprises the dosage amount administered. However, there are no blood levels of the NSAID in the immediate area of treatment. The forms of hyaluronic acid are thus cleared via the lymphatic system. Then the lymphatics pass the forms of hyaluronic acid, Applicants believe, to the blood system. Thus, the NSAIDs and forms of hyaluronic acid stay at the site to be treated for well in excess of 12 - 24 hours, a protracted stay.

Thus, over the period of treatment (for example, applications of effective non-toxic dosage amounts of compositions containing for example effective non-toxic dosage amounts of the NSAIDS and effective non-toxic dosage amounts of the sodium hyaluronate, 3 times a day for 2-4 weeks, transport the NSAIDS to to the epidermis to inhibit prostaglandin synthesis to enable the macrophages to "scavenge" the tumour cells and eliminate them. The end result is the successful treatment of the disease or condition at the site of trauma and/or pathology of the skin or exposed tissue, for example, the resolution of, the basal cell carcinoma, the precancerous, often recurrent, actinic keratoses lesions, fungal lesions, "liver" spots and like lesions (found for the most part in the epidermis), squamous cell tumours, metastatic cancer of the breast to the skin, malignancies and/or tumours in the skin, primary and metastatic melanoma in the skin, genital warts cervical cancer, and HPV (Human Papilloma Virus) including HPV of the cervix, psoriasis (both plaque-type psoriasis and nail bed

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psoriasis), corns on the feet and hair loss on the head of pregnant women, with complete disappearance of the disease or condition as the case may be, by topical therapy without resorting to surgery.

One of the formulations which we have employed successfully is a gel formulation comprising 3% diclofenac in 2.5% sodium hyaluronate formulated as follows:

Formulation 1 (3000 ml.)

Formula	Supplier	(LOT)	Amount	Percent
Glycerine	Life	1043	150 g (119 ml)	5
Benzyl Alcohol	Caledon	02517	90 g (86 ml)	3
Diclofenac Sodium	Prosintex	9113003	90 grams	3
Sodium Hyaluronate (MW 661,660)	Skymark	HG1003	75 grams	2.5
Sterile water balance	Baxter	AW4455	2795 ml.	

Procedure

- set up stirring apparatus using a 4 litre stainless steel beaker
- add water, Glycerine, and Benzyl Alcohol; stir to mix
- add Diclofenac Sodium and stir for 30 minutes
- then add the Sodium Hyaluronate and stir for 90 minutes
- initially, stir at a high torque but avoid splashing; as the gel thickens, stir at a lower torque.

The gel is then packaged in a tube or jar or other suitable container for use. Identification of suitable dosage amounts and how they are taken from the container may be provided with the container - for example squeeze "X" cm. of ribbon from the tube; fill spoon or spatula accompanying jar; (the spoon or spatula containing a predetermined dosage amount) then apply and rub into site of trauma and/or pathology (the dosage amount indicated will be such amount of the composition which comprises in excess of about 5 mg. of sodium hyaluronate per cm^2 (square centimeter) of skin or exposed tissue to which the dosage amount is to be applied. The amount of Diclofenac Sodium was determined in the same manner (having regard to the dosage amount required).

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Another such formulation is:

Formulation 2

Formula	Supplier	(LOT)	Amount	Percent
Methoxypolyethylene Glycol 350	Sigma	34F-0266	300 g.	20
Benzyl Alcohol	BDH	23797	15 g.	1
Diclofenac Sodium	Prosintex	9123012	45 g.	3
Sodium Hyaluronate (MW 679,000)	Skymart	HG 1004	37.5 g.	2.5
Sterile Water balance	Baxter	AW45R6	1200 ml.	

Procedure

- set up stirring apparatus using a 3 litre stainless steel beaker
- add water, Methoxypolyethylene Glycol 350, and Benzyl Alcohol and stir for 20 minutes to mix
- add Diclofenac Sodium and stir for 30 minutes to dissolve
- add Hyaluronate Sodium slowly and stir initially at a high speed, but avoid splashing
- after addition, stir at a slower speed for 90 minutes; the slower speed reduces the formation of air bubbles
- the result is a clear, transparent, viscous gel which is put into a container. Once again instructions are given for administration and if applicable measuring devices (to provide a premeasured dosage amount) accompany the container.

Still other formulations are:

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Formulation 3

3% Diclofenac in 2.5% HA Gel

Formula	Supplier	LOT	Amount	Percent
Sterile Water	Baxter	AW45K6	1200 ml	-
Methoxypolyethylene Glycol 350	Sigma	34F-0266	300G (273 ml)	20%
Benzyl Alcohol	BDH	23797	15G (14 ml)	1%
Diclofenac Sodium	Prosintex	9123012	45 g	3%
Sodium Hyaluronate MW 679,000	Skymart	HG 1004	37.5 g	2.5%

Procedure

- Set up stirring apparatus using a 2 liter stainless steel beaker,
- Add water, Methoxypolyethylene Glycol 350, and Benzyl Alcohol and stir for 20 minutes to mix,
- Add Diclofenoc Sodium and stir for 30 minutes to disolve,
- Add Hyularonate Sodium slowly and stir initially at a high speed, but avoid splashing,
- After addition, stir at a slower speed for 90 minutes,the slower speed reduces the formation of air bubbles,
- The result is a clear transparent, viscous gel which is poured into jars and tubes. Once again instructions accompany the container and where applicable appropriate devices for providing a premeasured amount of the composition accompany the container.

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Formulation 4

5% IBUPROFEN IN 3.0% HA GEL, 50 ml JAR

Formula	Supplier	LOT	Amount	Percent
Sterile Water	Baxter	AW45R6	196 ml	--
Meglumine	Falk	15684	11 g	5.5%
Ibuprofen	BDH	19/241	10 g	5%
Benzy Alcohol	BDH	23797	2 g	1%
Glycerin	BDH	2579	2 g	1%
Hyaluronate Sodium Mol Wt 661,600	Skymart	HG 1003	6 g	3%

PROCEDURE

- Set up stirring apparatus using a 300 ml stainless steel beaker,
- Add Sterile Water and Meglumine, and stir for 10 minutes,
- Add Ibuprofen and stir for 15 minutes,
- Add Benzyl Alcohol, followed by Glycerin and stir for 15 minutes,
- Finally, add Hyaluronate Sodium slowly and stir initially at a high torque to mix, but avoid splashing,
- As the gel thickens, stir at a slow speed for 90 minutes.

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Formulation 5

2% PIROXICAM IN 2.5% HA GEL

Formula	Supplier	LOT	Amount	Percent
Sterile Water	Baxter	AW45R6	200 ml	--
Meglumine	Falk	15684	8 g	4%
Piroxicam	AMSA	1-010	4 g	2%
Hyaluronate Sodium MW 661,600	Skymart	HG 1003	5 g	2.5%

PROCEDURE

- Set up stirring apparatus using a 300 ml stainless steel beaker,
- Add 200 ml of sterile water,
- Add 8 grams of Meglumine and dissolve,
- Very slowly add 4 grams of Piroxicam and stir for 20 minutes,
- Slowly add 5 grams of Hyaluronate Sodium and stir at high speed,
- Stir for 90 minutes at a slower speed

COMMENTS

- A clear yellowish transparent gel

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Formulation 6

5% IBUPROFEN CREAM, 50 ml JAR

OILY PHASE

Formula	Supplier	LOT	Amount	Percent
Liquid wax DICDD	Brooks	L-1424	450 g	15%
Brookswax D	Brooks	P-490	480 g	16%
Glycerin	BDH	109109/2578	150 g(119 ml)	5%
AQUEOUS PHASE				
Sterile Water	Baxter	AW45F1	1950 ml	--
Meglumine	Falk	15684	150 g	5%
Ibuprofen	BKH	19/241	150 g	5%
MW 200,00				
Sodium Hyaluronate	Skymart	001	45 g	1.5%
Preservative Suttocide A	Sutton	SH-107	9 g	0.3%

PROCEDURE

A - Add all the ingredients of the oily phase A into a 4 liter stainless steel beaker, melt at 55°c, finally heat to 75% when Aqueous Phase B is ready

B - Into a 3 liter stainless steel beaker, add 1950 ml water, set up, the stirring apparatus, add the Meglumine, stir to dissolve for 10 minutes,

- Slowly add Ibuprofen, stir to dissolve for 20 minutes,
- Very slowly add Sodium Hyaluronate and stir for one hour to dissolve all the Sodium Hyaluronate,
- Finally, heat to 75°C,with stirring for a total time of 30 minutes.

POUR B INTO A, both at a temperature of 75°C, slowly

- Remove the heat source and stir with a strong vortex for one hour,
- When the temperature has cooled down to 45°C add preservative Suttocide A,
- Continue stirring at a slower speed until the temperature is 35°C,
- At 35°C remove the propeller, pour into 50 ml jars.

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Formulation 7

1% DICLOFENAC IN 3% HA Gel, 50 ml jar

Quantity 3000ml

Formula	Supplier	LOT	Amount	Percent
Sterile Water	Baxter	AW45R6	2796ml	-%
Glycerin	BDH	2579	50g(71ml)	3%
Benzyl Alcohol	BDH	23797	45g(43ml)	1.5%
Liquid wax DICDD	Brooks	191-175	90 g	3%
Diclofenac Sodium	Prosintex	9113003	30 g	1%
Hyaluronate Sodium MW 679,000	Skymout	HG 1004	90 g	3%

PROCEDURE

- Set up stirring apparatus using a 4 liter stainless steel beaker.
- Add water, Glycerin, Benzyl Alcohol and Liquid wax DICDD and stir to mix thoroughly for 10 minutes
- Add Diclofenac Sodium and stir for 30 minutes to dissolve.
- Slowly add Hyaluronate Sodium, stirring at a high torque initially during addition.
- After addition stir at a slower speed for 90 minutes.
- A white opaque viscous gel is formed.

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Formulation 8

1% DICLOFENAC IN 3.0% HA Gel, 50 ml tube

Quantity 1500 ml

Formula	Supplier	LOT	Amount	Percent
Sterile Water	Baxter	AW45F1	1397 ml	-%
Glycerin	Life	1043	45g(36 ml)	3%
Benzyl Alcohol	Caledon	02517	22.5g(22ml)	1.5%
Liquid wax DICDD	Brooks	191-175	45 g	3%
Diclofenac Sodium	Prosintex	9113003	15 g	1%
Sodium Hyaluronate Mol. Wt. 661,600	Skymart	HG 1003	45 g	3%

PROCEDURE

- Set up stirring apparatus using a 3 liter stainless steel beaker.
- Add water, Glycerin, Benzyl Alcohol and Liquiwax DICDD, stir to mix for 10 minutes.
- Add Diclofenac Sodium and stir for 30 minutes to dissolve.
- Add Sodium Hyaluronate and stir for 90 minutes.

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Formulation 9

HYANALGESE CREAM (L)

50 ml tube
Quantity 3000 ml

FORMULA

A. Oily Phase	SUPPLIER	LOT	AMOUNT	PERCENT
Liquid Wax DICDD	Brooks/Amisol		450g	15.0%
Brookswax D	Brooks/Amisol		480g	16.0%
Glycerine	Amisol		150g	5.0%
B. Aqueous Phase				
Sterile Water	Baxter	AW4YA8	1950ml	-%
Meglumine	Falk		150g	5.0%
Sodium Hyaluronate MW 207,000	Skymart	P01	45g	1.5%
Ibuprofen	BDH		150g	5.0%
Suttocide A	Sutton		9.0g	0.3%

PROCEDURE

A. - Add all the ingredients of the oily phase into a 4 liter stainless steel beaker, melt at 55°C, finally heat to 75°C when aqueous phase is ready (at 75°C) to pour in.

B. - Into another 4 liter stainless steel beaker, add 1950 ml water.

- Set up the stirring apparatus and add the Meglumine
- Stir to dissolve with high torque, then slowly add Ibuprofen
- When the Ibuprofen is dissolved, slowly add Sodium Hyaluronate
- Stir cold for one hour to dissolve all the ingredients
- Finally heat to 75°C and stir thoroughly throughout a 30 minute period

MIX B INTO A

- Slowly pour B into A (both at 75°C) with stirring
- Immediately remove the hot plate (heat) and stir
- Stir with a strong vortex for one hour
- When the temperature is 45°C, add the preservative Suttocide A

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- Stir for about an hour to cool to 35°C
- At 35°C remove the propeller and pour into 50 ml tubes
- Pour 50 grams of the cream into each tube

1% BANAMINE IN 2.5% HA GEL

(L) XPB 041 Quantity 3000 ml

FORMULA

	SUPPLIER	LOT	AMOUNT	PERCENT
Sterile Water	Boxter	AW4SA2	2400 m	1--%
Sodium Hyaluronite MW 661,600	Skymart	HE1003	75g	2.5%
*Banamine, 100 ml vial	Scheing	O CNXB13	300 ml	1%
Banamine, 100 ml vial	Scheing	O CNXB12	300 ml	1%
			3000 ml	

(50 mg/ml) 600 = 30,000mg

= 30 grams Flunixin in 600 ml

*Banamine contains Flunixin Meglumine (50 mg Flunixin per ml)

or 83 mg Flunixin Meglumine

PROCEDURE

- Set up stirring apparatus using a 4 liter stainless steel beaker
- Add water, stir with a strong vortex, then add sodium Hyoluronate slowly
- Then immediately add the Banamine, stir the mixture for 4 hours.

One form of hyaluronic acid and/or salts thereof (for example sodium salt) and homologues, analogues, derivatives, complexes, esters, fragments, and sub-units of hyaluronic acid, preferably hyaluronic acid and salts and thereof, suitable for use with Applicant's invention is a fraction supplied by Hyal Pharmaceuticals Limited. One such fraction is a 15 ml vial of Sodium hyaluronate 20mg/ml (300mg/vial - Lot 2F3). The sodium hyaluronate fraction is a 2% solution with a mean average molecular weight of about 225,000. The fraction also contains water q.s. which is triple distilled and sterile in accordance with the U.S.P. for injection formulations. The vials of hyaluronic acid and/or

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salts thereof may be carried in a Type 1 borosilicate glass vial closed by a butyl stopper which does not react with the contents of the vial.

The fraction of hyaluronic acid and/or salts thereof (for example sodium salt) and homologues, analogues, derivatives, complexes, esters, fragments, and sub-units of hyaluronic acid, preferably hyaluronic acid and salts thereof, may comprise hyaluronic acid and/or salts thereof having the following characteristics:

a purified, substantially pyrogen-free fraction of hyaluronic acid obtained from a natural source having at least one characteristic selected from the group (and preferably all characteristics) consisting of the following:

i) a molecular weight within the range of 150,000-225,000;

ii) less than about 1.25% sulphated mucopolysaccharides on a total weight basis;

iii) less than about 0.6% protein on a total weight basis;

iv) less than about 150 ppm iron on a total weight basis;

v) less than about 15 ppm lead on a total weight basis;

vi) less than 0.0025% glucosamine;

vii) less than 0.025% glucuronic acid;

viii) less than 0.025% N-acetylglucosamine;

ix) less than 0.0025% amino acids;

x) a UV extinction coefficient at 257 nm of less than about 0.275;

xi) a UV extinction coefficient at 280 nm of less than about 0.25; and

xii) a pH within the range of 7.3-7.9.

Preferably, the hyaluronic acid is mixed with water and the fraction of hyaluronic acid has a mean average molecular weight within the range of 150,000-225,000. More preferably, the fraction of hyaluronic acid comprises at least one characteristic selected from the group (and preferably all characteristics) consisting of the following characteristics:

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i) less than about 1% sulphated mucopolysaccharides on a total weight basis;

ii) less than about 0.4% protein on a total weight basis;

iii) less than about 100 ppm iron on a total weight basis;

iv) less than about 10 ppm lead on a total weight basis;

v) less than 0.00166% glucosamine;

vi) less than 0.0166% glucuronic acid;

vii) less than 0.0166% N-acetylglucosamine;

viii) less than 0.00166% amino acids;

x) a UV extinction coefficient at 257 nm of less than about 0.23;

xi) a UV extinction coefficient at 280 nm of less than 0.19; and

xii) a pH within the range of 7.5-7.7

Applicants also propose to use sodium hyaluronate produced and supplied by LifeCore™ Biomedical, Inc., having the following specifications:

Characteristics	Specification
Appearance	White to cream colored particles
Odor	No perceptible odor
Viscosity Average Molecular Weight	< 750,000 Daltons
UV/Vis Scan, 190-820nm	Matches reference scan
OD, 260nm	< 0.25 OD units
Hyaluronidase Sensitivity	Positive response
IR Scan	Matches reference
pH, 10mg/g solution	6.2 - 7.8
Water	8% maximum
Protein	< 0.3 mcg/mg NaHy
Acetate	< 10.0 mcg/mg NaHy
Heavy Metals, maximum ppm	

As	Cd	Cr	Co	Cu	Fe	Pb	Hg	Ni
2.0	5.0	5.0	10.0	10.0	25.0	10.0	10.0	5.0

Microbial Bioburden	None observed
Endotoxin	< 0.07EU/mg NaHy

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Biological Safety Testing		Passes Rabbit Ocular Toxicity Test

Another form of sodium hyaluronate is sold under the name Hyaluronan HA-M5070 by Skymart Enterprises, Inc. having the following specifications:

Specifications' Test Results	
Lot No.	HG1004
pH	6.12
Condroitin Sulfate	not detected
Protein	0.05%
Heavy Metals	Not more than 20 ppm
Arsenic	Not more than 2 ppm
Loss on Drying	2.07%
Residue on Ignition	16.69%
Intrinsic Viscosity	12.75 dl/s (XW: 679,000)
Nitrogen	3.14%
Assay	104.1%
Microbiological Counts	80/g
E. coli	Negative
Mold and Yeast	Not more than 50/g

Other forms of hyaluronic acid and/or its salts, and homologues, derivatives, complexes, esters, fragments and sub units of hyaluronic acid may be chosen from other suppliers, for example those described in prior art documents provided the form of hyaluronic acid chosen is suitable for transport of the medicine.

The following references teach hyaluronic acid, sources thereof, and processes for the manufacture and recovery thereof which may be suitable.

United States Patent 4,141,973 teaches hyaluronic acid fractions (including sodium salts) having:

> "(a) an average molecular weight greater than about 750,000, preferably greater than about 1,200,000 - that is, a limiting viscosity number greater than about 1400 $cm^3/g.$, and preferably greater than about 2000 $cm^3/g.$;

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(b) a protein content of less than 0.5% by weight;

(c) ultraviolet light absorbance of a 1% solution of sodium hyaluronate of less than 3.0 at 257 nanometers wavelength and less than 2.0 at 280 nanometers wavelength;

(d) a kinematic viscosity of a 1% solution of sodium hyaluronate in physiological buffer greater than about 1000 centistokes, preferably greater than 10,000 centistokes;

(e) a molar optical rotation of a 0.1 - 0.2% sodium hyaluronate solution in physiological buffer of less than -11×10^3 degree - cm^2/mole (of disaccharide) measured at 220 nanometers;

(f) no significant cellular infiltration of the vitreous and anterior chamber, no flare in the aqueous humour, no haze or flare in the vitreous, and no pathological changes to the cornea, lens, iris, retina, and choroid of the owl monkey eye when one milliliter of a 1% solution of sodium hyaluronate dissolved in physiological buffer is implanted in the vitreous replacing approximately one-half the existing liquid vitreous, said HUA being

(g) sterile and pyrogen free and

(h) non-antigenic."

Canadian Letters Patent 1,205,031 (which refers to United States Patent 4,141,973 as prior art) refers to hyaluronic acid fractions having average molecular weights of from 50,000 to 100,000; 250,000 to 350,000; and 500,000 to 730,000 and discusses processes of their manufacture.

In order to determine the blood levels in patients using formulations made according to embodiments of the invention, a study of the pharmacokinetic profiles of two topical diclofenac formulations after repeat dosing were undertaken.

One such product was the product Voltarol Emulgel marketed in the United Kingdom by Geigy. The other was a Diclofenac preparation in Hyaluronic Acid.

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This was an open, repeat dose, crossover comparison using a randomized balanced block in six healthy volunteers.

The study consisted of administration with one, two week period in between periods, each period lasting fourteen days. The test articles applied were for the first six days of each period and the seventh day was study day during which the final application is made and blood samples taken.

The approximate duration of the study including pre and post study screening was six weeks.

Doses

Diclofenac (3.0%) with Hyaluronic Acid (2.5%)
Dose: Approximately 2 g, three times daily
Route: Topical

(W1)Voltarol Emulgel, Diclofenac diethylammonium salt 1.16g aqueous gel (Geigy)
Dose: Approximately 2 g, three times daily
Route: Topical (W1)

ADMINISTRATION: to suitable patients

Subjects applied one of the designated test articles topically to the calves and massaged into the skin, in a dose of approximately 2 g per application three times a day for six consecutive days. The size of a 2g dose was prepared by comparison with a silicone example given to each subject.

On the seventh day, the cream was applied once, in the same manner as before, under the supervision of the staff of the Clinical Investigation Unit.

After a washout period of one week the procedure was repeated with the alternate test article.

The following were the results of the tests:

(H = hyaluronic acid formulation)
(V = Voltarol Emulgel)

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PERIOD 1

All concentrations ng ml - 1

SUBJECT	TIME POINT (hours)											
	0	0.25	0.5	1	2	3	4	5	6	8	10	12
H-1	10.3	7.1	6.4	ND	ND	5.4	6.5	5.1	ND	ND	ND	ND
H-2	ND	5.1	ND	5.1	ND	ND	ND	ND	ND	5.1	ND	ND
ND	ND	ND	5.5	5.2	ND	ND	ND	ND	ND	ND	ND	V-3
ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	H-4
ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	V-5
ND	ND	ND	ND	ND	ND	ND	8.4	ND	ND	ND	ND	V-6

ND = NONE DETECTED (>5.0 ng ml^{-1})

PERIOD II

All concentrations ng ml -1

SUBJECT	TIME POINT (hours)											
	0	0.25	0.5	1	2	3	4	5	6	8	10	12
V-1	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
V-2	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
H-3	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
V-4	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
H-5	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
H-6	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND

ND = NONE DETECTED (>5.0 ng ml^{-1})

Other tests were undertaken to determine blood levels comparing Proflex (a formulation containing Ibuprofen) and the following formulation containing hyaluronic acid and Ibuprofen.

HYANALGESE CREAM (L) X PB 022

- 50 ml tube

Quantity 3000 ml

FORMULA A. Oily Phase	SUPPLIER	LOT	AMOUNT	PERCENT
Liquid Wax DICDD	Brooks/Amisol		450g	15.0%
Brookswax D	Brooks/Amisol		480g	16.0%
Glycerine	Amisol		150g	5.0%
B. Aqueous Phase				
Sterile Water	Baxter	AW4YA8	1950ml	-%
Meglumine	Falk		150g	5.0%
Sodium Hyaluronate MW 207,000	Skymart	P01	45g	1.5%
Ibuprofen	BDH		150g	5.0%
Suttocide A	Sutton		9.0g	0.3%

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The following were the results

(A) PROFLEX

SUBJECT Number PD	Time after administration (Hours) 0	0.25	0.5	1	2	3	4	5	6	8	10	12	
1	ND	0.41	0.37	0.37	0.32	0.30	0.27	0.27	0.24	0.37	0.31	0.31	0.16
2	ND	0.12	0.12	0.08	0.11	0.12	0.12	0.07	0.08	0.09	0.08	ND	0.06
3	ND	0.09	0.08	0.07	ND	ND	ND	ND	ND	ND	ND	ND	ND
4	ND	0.12	0.14	0.16	0.11	0.11	0.25	0.24	0.17	0.13	0.16	0.11	0.13
5	ND	0.14	0.19	0.19	0.15	0.16	0.16	0.14	0.12	0.11	0.13	0.10	0.07
6	ND	0.11	0.09	0.09	0.06	0.07	0.05	0.05	0.05	ND	ND	ND	ND
Mean	0.00	0.17	0.17	0.16	0.13	0.13	0.14	0.13	0.11	0.12	0.11	0.09	0.07
S.D.	0.00	0.12	0.10	0.11	0.10	0.10	0.10	0.10	0.08	0.13	0.11	0.12	0.06

(B) HYALURONIC ACID AND IBUPROFEN

SUBJECT Number PD	Time after administration (Hours) 0	0.25	0.5	1	2	3	4	5	6	8	10	12	
1	ND	0.11	0.11	0.12	0.08	0.08	0.09	0.11	0.12	0.08	0.11	0.16	0.14
2	ND	0.22	0.21	0.26	0.17	0.24	0.24	0.25	0.23	0.19	0.19	0.20	0.14
3	ND	0.17	0.10	0.12	0.09	0.08	0.07	0.06	ND	0.06	0.26	0.09	0.05
4	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
5	ND	0.17	0.16	0.16	0.12	0.09	0.10	0.11	0.10	0.09	0.10	0.07	ND
6	ND	0.07	0.07	0.09	ND	ND	ND	ND	ND	ND	ND	ND	ND
Mean	0.00	0.12	0.11	0.13	0.08	0.08	0.08	0.09	0.08	0.07	0.11	0.09	0.06
S.D.	0.00	0.08	0.07	0.08	0.06	0.08	0.08	0.09	0.09	0.07	0.10	0.08	0.07

ND None detected <0.05 µg/ml

The above clearly indicates that the blood levels are much less using hyaluronic acid to administer the NSAID.

PRELIMINARY REPORT

A trial was conducted using a gel composition (Number 109) comprising 3% Diclofenac in 2.5% Hylauronic Acid as previously described and a composition containing Diclofenac sodium salt 3% but not including any form of hyaluronic acid. (Number 112) The trial was conducted with 60 patients who were randomly assigned to test preparations number 109 or 112. The trial has not been completed as yet but so far 31 patients have finished the protocol. Patients were diagnosed:

4 Rheumatoid arthritis of the knee

8 Myofascial trigger points in the M.trapezius area

12 Periarthropathies of knee without effusion

7 Periarthropathies with effusion in the knee joint

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The 31 patients were aged 22-75 years (27 females, 4 males). All patients were hospitalized. Patients entering the trial were thoroughly examined and type of extraarticular or articular rheumatism assessed.

On day 1 baseline pain was assessed on the 10 cm visual analogue scale (VAS) and pain measurement of the quantititative pain sensitivity using a pressure tolerance meter (PTM) were performed. Then test gel - approximately 2.g - was massaged on to the skin of maximum pain. Gels were applied 3 times daily.

0.5, 1, 1.5, and 2 hours after morning application measurements of pain sensitivity were carried out and values recorded.

This procedure was countinued on day 2, 3 and 4; measurements (VAS and PTM) of pain severity were done on day 1, 2 and 4.

Prior of the beginning of the study and at the end on day 4, physician's global assessment, assessment of swelling, tenderness and limitation of movement were recorded.

As the study is ongoing statistical evaluation is not yet available. For further details see Table 1.

TABLE 1

	Composition	Composition
Reaction	109, n = 16	112, n - 15
Good Alleviation of pain	13	8
Moderate Alleviation of pain	2	2
No Alleviation of pain	1	5

From the data recorded we have concluded that the patients to whom composition 109 was administered did better in terms of earlier and longer lasting analgesic effect (up to 4 hours) than the 112 composition especially in patients with myofascial trigger points and with periarthropathies of the knee joints without effusions. Neither composition 109 nor

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composition 112 treated patients showed any effect on swelling if any swelling exist at all. Systemic side effects have not been observed; one patient to whom composition 112 was administered showed reddening of the skin on the site of application.

Any intake of system NSAIDS, corticosteroids and other analgesics was not allowed one week before and during the trial.

EXAMPLES

The following examples are offered to illustrate uses of Applicants' invention.

Example 1

A male patient had a number of lesions (basal cell carcinoma), including one on his forehead which was a combination of major "horny epithelium" and some degree of ulceration. After continuous treatment with Formulation 1 (several times per day for several weeks of dosage amounts squeezed from tubes as ribbons of composition), the lesions showed epithelialization, no hemorrhagic areas, and no initiated areas (as they were in the past without our treatment). The "horny epithelium" and ulceration of the forehead lesion were also gone. The patient had a complete successful response with the formulation. All basal cell carcinoma lesions had been resolved and disappeared. There has been no recurrence.

Example 2

60 year old male tennis player had sore elbow and basal cell carcinoma on forearm proximate sore elbow. Patient tried Formulation 1 to abate pain in tennis elbow. (Dr. Falk was not treating this patient for anything at the time, did not know of the basal cell proximate the elbow and merely offered the formulation for pain relief of the elbow instucting the patient to squeeze a ribbon of the composition and apply and rub into the sore elbow). However, the formulation "spilled" over onto the Patient's basal cell carcinoma. Patient was planning to have basal cell carcinoma

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removed surgically by another doctor, but when the patient returned to see the doctor, the basal cell carcinoma was disappearing (because of spill-over of Formulation 1). Dr. Falk was then advised and treatment was now undertaken by Dr. Falk with direct application of Formulation 1 to the lesion 3 times a day for two additional weeks. After two weeks, the basal cell carcinoma disappeared. There has been no recurrence.

Example 3

Male, mid to late 40's had severe basal cell carcinoma on left temple. Doctors recommended its removal by surgery. However, the surgery would have been risky because of the lesion's proximity to facial nerves.

Patient saw Dr. Falk who gave him Formulation 2 to be applied in dosage amounts 3 times daily.

After 14 days, 75% of the lesion was gone. Surgery was postponed and the treatment was continued. Application of dosage amounts of Formulation 2 was continued for an additional two weeks. At the end of the 2- week period, the lesion was completely resolved and disappeared without any surgery being required. There has been no recurrence.

Example 4

Male, early 40's, had recurrent actinic keratoses lesion on his right temple. Early attempts at removal by third parties involved the application of liquid nitrogen (twice) without final resolution. The lesion kept recurring. The patient was sent to Dr. Falk who treated the lesion with Formulation 1 with applications of dosage amounts 3 times daily for 7 days. After 7 days, the lesion was completely resolved with no subsequent recurrence.

Example 5

A male patient suffering from kyphosis suffered from constant back pain. Taking analgesics orally and rubbing back preparations onto his back did little to alleviate the back pain. When NSAIDs in hyaluronic acid (sodium hyaluronate)

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were applied directly to the back, the back pain eased and disappeared.

With indomethacin (dissolved in N-methyl glucamine) and naproxen both dissolved in hyaluronic acid, the patient experienced some side effects. However, with Toradol™ (the [+/-] form tromethamine salt of ketorolac - a prostaglandin biosynthesis inhibitor and analgesic and anti-inflammatory, the back pain eased and disappeared for some time and there were no side effects. The compositions were applied generously onto the sites of back pain.

Example 6

A male patient with basal cell carcinoma was first treated by an oncologist who attempted to surgically excise the lesion (without success) and then irradiated the lesion again without success. The patient then attended before Dr. Falk who applied Applicant's formulation (diclofenac with sodium hyaluronate and excipients). Application was made three times daily for about a month and the lesion disappeared. Some excoriation anterior and slightly superior developed over the last two weeks but was cleared by the application of hyaluronic acid by itself.

This resolution clearly indicates that even with prior applications of unsuccessful therapies (surgery and irradiation), Applicant's formulations can be used successfully.

Example 7

In another patient, a drug (methotrexate) was carried in hyaluronic acid and applied topically to a patient with psoriasis. The formulation was absorbed and the psoriasis cleared.

Example 8

A patient with dermal (skin) metastases in a fibratic scar form and metastatic cancer in the form of musculoskeletal involvement in her thorax.

On topical application of our formulation comprising diclofenac (Voltaren) in hyaluronic acid (sodium hyaluronate),

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her pain decreased dramatically and her skin and boney involvements steadily improved.

TOPICAL DICLOFENAC ACID 3% IN HYALURONIC ACID GEL (2.5%) BASE

A practitioner reviewed the effectiveness of topical Diclofenac Acid 3% in hyaluronic acid gel (2.5%) base in acute traumatic injuries of no longer than 3 days duration. The cases were all in the spectrum of ages between 18 and 65. Normal exclusion criteria were followed regarding exclusion of pregnancy, aspirin or N.S.A.I.D., allergies or active peptic ulceration.

As an overall, the following impressions were gained from 30 cases:

1. The topical H.D.(composition comprising sodium hyaluronate and diclofenac) had an obvious analgesic action with onset occurring rapidly within one hour; this is a phenomenon not obviously seen with other non-steroidals that we have used.
2. There was a very definite patient acceptance of the gel as a form of treatment, being logical, easy to apply, without local or systemic side effects, rapid absorption with no staining of clothing.
3. The anti-inflammatory action was equivalent on a "guestimate" based on experience of similar injuries to oral N.S.A.I.D.s, without the threat or risk of side effects.

In summary, compared with other topical N.S.A.I.D.s the analgesic effect is distinct, the anti-inflammatory is equal to oral N.S.A.I.D.s and the patients' acceptance is far superior to any other diclofenac or piroxicam topical that the practitioner evaluated.

Following the practitioner's basic preamble regarding the parallelism of topical N.S.A.I.D.s and topical steroids,the practitioner has used the former in contact dermatitis, insect bites and U.V. erothema, all with very positive effects, again pointing direction to trials of a double blind nature in these fields.

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CHRONIC CONDITIONS - EVALUATIONS

2.5% HYALURONIC ACID WITH 3% DICLOFENIC ACID (HD)

Patients Initials (M) or (F)	Date Of Birth	File No.	Diagnosis	Comments on Outcome	Positive (P) Negative (N) Unable to Comment (U)
LA (M)	11.04.56		Hyper-aesthesia	Severe discomfort following extensive surgery to dorsal spine with insertion of rods in 1989. Even contact with clothes produced significant discomfort. Initially treated with EMLA with only transient anaesthetic results, however even after 3 days treatment with Hyal diclofenac acid noticed marked decrease in supersensitivity which has continued for at least 4 weeks while still using gel.	P Example of peripheral action on super-sensitization of nerve ending queried.
KB (F)	08.06.58		Chronic chondro-malacia perhaps dating back to 1976.	Treated right knee which was worse initially and was amazed at the response, then started to treat left knee that was not so painful, again with positive response. Here we have a built-in control.	P
DB (F)			Chronic neurogenic pain in ankle with associated dysaesthesia.	Initially felt some improvement which was not continued although initially quite positive - query placebo reaction.	N
DC (F)	07.11.51		Chronic back pain - query due to facet syndrome or trigger points, really diagnosis uncertain.		N

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CHRONIC CONDITIONS - EVALUATIONS

HYALURONIC ACID WITH 3% DICLOFENAC ACID (HD)

Patients Initials (M) or (F)	Date Of Birth	File No.	Diagnosis	Comments on Outcome	Positive (P) Negative (N) Unable to Comment (U)
CC	18.01.25		Chronic capsulitis right hip right knee	Definite effect over knee where application to target distance short. No obvious effect over hip.	P
AG (F)	07.11.58		Myositis in rhomboids muscles following motor vehicle accident	Initially given placebo in error, only marginal or minimal effect, if any. Found active to be effective while being used, did not cure condition which needed trigger point therapy.	P
CH (F)	22.08.61		Chronic relapsing tendonitis right elbow	No significant effect, nor has aggressive therapy since including injection with cortisone and numerous opinions.	N
SH (F)	16.07.55		Tendonitis and myositis	Control of tendonitis while using preparation. Is now back at work.	P
DM (M)	17.06.47		Neuronitis	This patient has a very unusual pain in his left groin following nerve injury, with the use of preparation noticed decrease in pain sensation while on medication. Hyperaesthesia altered although pain (which may be phantom) still present.	U
PJ	15.06.45		Capsulitis of right wrist	Symptoms improved 50% while using Hyal diclofenac acid, however, on discontinuation pain reappeared. Exact etiology uncertain.	U

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CHRONIC CONDITIONS - EVALUATIONS

HYALURONIC ACID WITH 3% DICLOFENAC ACID (HD)

Patients Initials (M) or (F)	Date Of Birth	File No.	Diagnosis	Comments on Outcome	Positive (P) Negative (N) Unable to Comment (U)
DJ (F)			Dorsal myositis	Control while using gel equal and with less side effects than tiger balm. Controlled symptoms while using medication. Exact diagnosis as to cause of myositis uncertain.	P
DK (F)	27.08.38		Severe capsulitis left shoulder	This patient has had capsulitis left shoulder for many years and treated with only transient relief with cortisone injections, poor relief with topical piroxicam. Was started on topical diclofenac acid and noticed relief of pain in 20 minutes continuing for 4 - 6 hours. See letter March 11/92.. At present is using H.D. regularly, has found it to be useful in other areas of chronic pain. Is President North American Chronic Pain Association, has good insight into medication and placebos etc. Has two D.C.S.. implants.	P Extremely rewarding case

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CHRONIC CONDITIONS - EVALUATIONS

HYALURONIC ACID WITH 3% DICLOFENAC ACID (HD)

Patients Initials (M) or (F)	Date Of Birth	File No.	Diagnosis	Comments on Outcome	Positive (P) Negative (N) Unable to Comment (U)
JL (M)	10.12.45		Chronic myositis secondary to query facet syndrome	Pain has failed to respond to many aggressive treatments.	N
RMC(F)	13.06.57		Neuronitis following facet rhizotomy with resulting pain in her back	It is a difficult case with considerable overlay, she obtained some relief with H.D., would estimate 30-40% Interestingly hyperanaesthesia was decreased.	U
RM (F)	20.08.52		Chronic capsulitis	Using H.D. significant improvement in pain while used, on stopping treatment recurrence of pain, needed intra-articular cortisone.	P
GM (F)			Sub-acute tendonitis right ankle	Rapid resolution of pain within one day and positive return of function.	P
PM (F)	20.09.46		Acute on chronic osteo-arthritis of first metatarsal phalyngeal joints	Rapid analgesic response with rapid settlement.	P
DN (F)	10.03.44		Chronic fasciaitis of feet	Excellent response to application of H.D. with occlusion. Had failed to respond to oral N.S.A.I.D.s and physiotherapy. Query positive result due to short application target distance in a vascular tissue.	P

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CHRONIC CONDITIONS - EVALUATIONS

HYALURONIC ACID WITH 3% DICLOFENIC ACID (HD)

Patients Initials (M) or (F)	Date Of Birth	File No.	Diagnosis	Comments on Outcome	Positive (P) Negative (N) Unable to Comment (U)
BP (F)	04.03.20		Severe chronic arthritis of the knee. Unable to take oral N.S.A.I.D.s	Initially one knee treated with such good results that both knees treated, see letter. Not only did pain decrease but marked swelling around knees. Significant relief of pain and increase in movement as a result of this and perhaps reduction of swelling. Intrestingly has severe superficial varicose veins, developed thrombophlebitis around right knee and the area treated by chance showed far less redness and tenderness than the thrombophlebitis below this area.	P Side effects-non/Incidental resolution of area of thrombophlebitis below area of treatment
SP (M)	06.11.48		Idiopathic diffuse capsulitis of hands	Has had similar episodes with poor response to many treatments including N.S.A.I.D.s per os	U
WS	04.06.45		Chronic neuronitis due to injury to lateral cutaneous nerve of thigh	Has been exposed to numberous treatments including tow attempts of surgery without effect. There is decrease in hyperaesthesia but no change in pain.	U
MS (F)	04.06.28		Chronic capsulitis	Failed to respond to number of treatments, good background resolution of pain, however, still had acute pain with certain movements.	P

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CHRONIC CONDITIONS - EVALUATIONS

HYALURONIC ACID WITH 3% DICLOFENAC ACID (HD)

Patients Initials (M) or (F)	Date Of Birth	File No.	Diagnosis	Comments on Outcome	Positive (P) Negative (N) Unable to Comment (U)
IS (F)	15.01.48		Chronic capsulitis	Had failed to respond to numerous treatments including oral and topical N.S.A.I.D.s Using H.D there was equivalent control of pain as with other therapies which lasted while medication was used. Referred for surgical opinion.	P
GS (F)	26.03.47		Chronic tendo-sinovitis tion.	Oral diclofenac acid discontinued due to gastritis and also history of ulceration. Control using H.D. equal to or better than oral N.S.A.I.D.s.	P
VK (F)	01.01.39		Chronic tendonitis	Good relief of pain and tenderness while using H.D. however on discontinuation of gel symptoms returned, treated with intramuscular steroids.	P for pain N for resolution
GH (M)	03.11.21		Acute on chronic osteo-arthritis left hand	In view of age and general parous medical condition, ideal for topical. Had been previously on topical piroxicam for left shoulder capsulitis.	P Commented on better absorption compared to topical piroxicam

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CHRONIC CONDITIONS - EVALUATIONS

HYALURONIC ACID WITH 3% DICLOFENAC ACID (HD)

Patients Initials (M) or (F)	Date Of Birth	File No.	Diagnosis	Comments on Outcome	Positive (P) Negative (N) Unable to Comment (U)
JA (M)	06.02.58		Severe post-traumatic and surgical osteo-arthritis of left leg with staples. Poor result to oral N.S.A.I.D.s also gastric irritation.	Produced good superficial analgesia especially where staples were irritating sub-cutaneous tissue, little effect on deeper, severe osteoarthritic pain of knee. This pain was of consider-able severity, needing nar-cotics.	P
IM (M)	30.11.51		Chronic superficial myositis	Severe rhomboid inflammation right side, treated with H.D., very definite improve-ment in pain and tenderness.	P
TK (F)	23.04.70		Acute on chronic capsulitis due to sports injury right hand	Excellent rapid analgesic followed by anti-inflammatory response in young women who could not take oral N.S.A.D.s due to past gastritis.	P
AD (F)	03.01.49		Chronic diffuse pain thought to be myositis	Poor response to H.D. After intensive investigation and numerous consultations and treatment, pain still un-diagnosed and unresponsive.	N
NH (F)	25.03.25		Subacute capsulitis right ankle	Excellent response analgesic and anti-inflammatory-wise within a few days. Marked clinical improvement. In view of this patient's parous general medical con-dition and hypertension, not suitable for oral NSAIDs.	P

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CHRONIC CONDITIONS - EVALUATIONS

HYALURONIC ACID WITH 3% DICLOFENAC ACID (HD)

Patients Initials (M) or (F)	Date Of Birth	File No.	Diagnosis	Comments on Outcome	Positive (P) Negative (N) Unable to Comment (U)
MD	18.04.34		Subacute rheumatoid arthritis	Had failed to respond to oral N.S.A.I.D.s, which caused gastritis, tried on topical piroxicam with negative effects. Negative response to H.D.	N
MW (F)	07.05.46		Heberden's nodes, painful, swollen causing difficulty in movement	Very slow positive outcome, initially improvement in pain followed by reduction in swelling. Etiology of this condition is unknown, partly genetic. Would have been interesting to treat alternate digits, plus or minus thermographic confirmation.	P
LP (F)	20.07.23		Acute on sub-acute osteo-arthritis of the hands with Heberden's nodes	Initially treated with Idarac, poor response overall, some improvement in generalised arthritis of hands but none on Heberden's nodes. Pain flared on stoppiny Idarac due to gastritis. Started on H.D., especially favourable results with subsidence of tenderness of nodes and settling of arthritis. Interestingly enough, no flare up on discontinuation after one month.	P

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CHRONIC CONDITIONS - EVALUATIONS

HYALURONIC ACID WITH 3% DICLOFENAC ACID (HD)

Patients Initials (M) or (F)	Date Of Birth	File No.	Diagnosis	Comments on Outcome	Positive (P) Negative (N) Unable to Comment (U)
JG (F)	24.11.50		Post facet rhizotomy hyper-aesthesia, with marked pain and hyper-aesthesia between scapulae	Had failed to respond to oral N.S.A.I.D.s and E.M.L.A. Application of H.D. improved the surface pain significantly but had no effect on the deeper pain. My impression was that the deeper pain was due to section of the facet nerve and beyond the reach of the topical medication. There is little doubt that the skin sensitivity was decreased.	U
SW (F)	10.09.39		Knee pain due to chondro malacia	Upset in past due to oral N.S.A.I.D.s., also hyper-tension made one loathe to use this medication with serum levels. Good anal-gesic and anti-inflammatory action, however on discon-tinuation pain flared. Seen for arthroscopic surgery with relief of pain.	P (Effec-tive while being used) Condition only cured by surgery

* Two types of pain-response in only one

1. Interestingly in the whole series, there was not one case of local side effects and as expected from past studies, no general or systemic. Since this report was prepared we have had one case of mild folliculitis which responded to discontinuation of treatment, will rechallenge.

2. A number of patients commented that they felt the gel improved the texture and softness of their skin, and commented that it was messy or stained their clothes.

3. In one case of topical thrombophlebitis where the inflamed vein crossed the area of treatment, the vein in the area of treatment improved while that outside at a distance did not. Again, similar to using oral N.S.A.I.D.s. ***.

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Photographs were taken of patients with basal cell carcinoma Figures 1-6 photographs, and of mice with tumors induced in the skin of the hind legs (Figure 7 photographs). The patients were treated by using combinations of NSAIDS, (non-steroidal anti-inflammatory drugs) and hyaluronic acid (including sodium hyaluronate) according to the invention (3% diclofenac in 2.5% sodium hyaluronate gel base). Each of the six sets of Figures made up of photographs of the different persons should include a legend describing or explaining each picture as follows:

Legend for Figures 1A and 1B should read:

Patient : W.D., male, 82 years
Diagnosis: Basal cell carcinoma
Treatment: NSAIDS plus HA gel. 3 times per day
Figure 1A: June, 1991
Figure 1B: December, 1991

Legend for Figures 2A and 2B should read:

Patient : M.F., male, 45 years
Diagnosis: Basal cell carcinoma
Treatment: NSAIDS plus HA gel. 3 times per day
Figure 2A: January, 1992
Figure 2B: April, 1992

Legend for Figures 3A, 3B, 3C and 3D should read:

Patient : H.A., male, 82 years
Diagnosis: Basal cell carcinoma
Treatment: NSAIDS plus HA gel. 3 times per day
Figure 3A: January 26, 1992
Figure 3B: March 16, 1992
Figure 3C: January 26, 1992
Figure 3D: March 16,1992

Legend for Figures 4A, 4B, 4C and 4D should read:

Patient : R.F., male, 64 years
Diagnosis: Basal cell carcinoma
Treatment: NSAIDS plus HA gel. 3 times per day
Figure 4A: January 26, 1992
Figure 4B: March 16, 1992
Figure 4C: January 26, 1992
Figure 4D: March 16, 1992

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Legend for Figures 5A, 5B, 5C and 5D should read:

Patient : R.W., male, 86 years
Diagnosis: Basal cell carcinoma
Treatment: NSAIDS plus HA gel. 3 times per day
Figure 5A: January 26, 1992
Figure 5B: March 16, 1992
Figure 5C: January 26, 1992 untreated
Figure 5D: March 16, 1992 untreated

Legend for Figures 6A, 6B and 6C should read:

Patient : E.D., female, 70 years
Diagnosis: Basal cell carcinoma
Treatment: NSAIDS plus HA gel. 3 times per day
Figure 6A: April 20, 1992
Figure 6B: May 13, 1992
Figure 6C: July 7, 1992

The Legend for Figure 7 (Figures 7A and 7B) relate to:

Mouse Strain: DBA_2
Tumour: p815
Figure 7A: control, 19 days
Figure 7B: Novantrone plus HA gel 19 days

The mice shown in Figures 7A and 7B had tumours induced in the skin of their hind legs and dosage amounts (2ml) of Novatrone (10 mg. per dosage amount) (MITOXANTRONE (t.m.) and 2.5% sodium hyaluronate were applied (rubbed onto) the skin at the site of the pathology. The tumours reduced in size (See Figure 7B) clearly illustrating the percutaneous delivery of the medicine by the hyaluronic acid. (See Figure 7).

The following additional comments are made with respect to the patients.

With respect to R.W. and Figure 5, the reader will note in Figures 5a and 5c the patient suffered from basal cell carcinoma on his back (Figure 5c) and his temple (Figure 5a). Because of the age of the individual (86) the basal cell carcinoma on his back could not be reached by him for application of the medication. Thus the basal cell carcinoma in 5c remained untreated and grew (see Figure 5d). However, the portion indicated in 5a on his temple could be reached and after application of the basal cell carcinoma formulation to

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the temple and forehead the results are as in 5b; the basal cell carcinoma is disappearing. Thus, the gentlemen's own method of treatment acted as a control.

With respect to R.F. and Figures 4, two areas of basal cell carcinoma in need of treatment are shown by the arrows in Figures 4a and 4c and the results are shown in Figures 4b and 4d as indicated by the arrows after treatment with Applicant's invention.

With respect to H.A., male, and Figures 3, Figures 3 indicates two areas of basal cell carcinoma by the arrows, close-ups of which are shown in Figures 3a and 3c. After treatment with the NSAIDS and HA gel three times a day for the period between January 26, 1992 and March 16, 1992 the basal cell carcinoma is clearing as per Figures 3b and 3d.

The same is true with respect to male M.F. and Figures 2 which appears clear in the photographs (see Figure 2a and the response shown in Figure 2b).

With respect to male, W.D. and Figures 1, the upper lesion in Figure 1a (indicated by the upper arrow) is gone after treatment with Applicant's invention (See Figure 1b) and the two lower lesions in Figure 1a are well on their way to disappearing (See Figure 1b).

With respect to female D and Figure 6, the lesion was left untreated for a long period and gradually encompassed her eye. Surgery could not be undertaken without jeopardizing the eye. By applying Applicant's invention (dosage amounts) over a prolonged period, the basal cell carcinoma has constantly decreased in size.

With respect to Figure 7, (7a) shows mice having tumors in the skin induced in their hind legs. After continuous applications to the shaved hind legs having the tumors in the skin by rubbing in dosage amounts by Applicant's invention, the tumors have decreased in size. (See Figure 7b)

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The effect of Hyaluronic acid as a drug carrier of anti-cancer agent (5-FU) 5-Fluoracil was also studied.

(Intratumour injection study)

B. EXPERIMENTAL MODEL (2)

1. Method and Material

a. Animal : Fisher 344 rat, male
200-250g

b. Tumor model
Fisher Bladder Carcinoma
Tumor (2mm viable tumor fragment) was transplanted subcutaneously on the right frank by trocar

c. Treatment was started when tumor size is about 1.5 cm.
(2 weeks after implantation.)

1. These drugs were administrated by intratumor injection. (right frank)
At the same time, injection into normal skin (left frank) was carried out similarly.

Group A :	H-5-FU 5mg/kg +	saline	/0.3ml (i.t.)
B :	H-5-FU 5mg/kg +	HA 15 mg/kg	/0.3ml (s.c.)

3H-FU without or with HA was injected as a single dose (0.3ml) into the center of the tumor (on the right frank) with a 30 gause needle. At the same time, injection into normal skin (on the left frank) was carried out similarly.

The tumor and skin was then removed at different times (1h,6hr) for counting radioactivity of the remaining content in the tissue.

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2. Result

All the results were expressed as Mean + S.E. under the following headings:

TUMOR TISSUE (left hand portion of the graph)	NORMAL SKIN (Right hand portion of the graph)
5-FU+HAgroup(n=4)	5-FU+HAgroup(n=4)
5-FU group(n=4)	5-FUgroup(n=4)

(See Figure 4' of Page 4/12)

3. Conclusion

1. In 5-FU HA group radioactivity was accumulated and retained in the tumor tissue for a long period, whereas rapid clearance was demonstrated in normal tissue. (skin)

2. In 5-FU group, radioactivity immediately disappeared from the tumor or the normal tissue by diffusion, primarily into blood capillaries.

---- 5FU can traverse freely between the interstitial space and blood capillary.

The Effect of Hyaluronic Acid as a Drug Carrier in Target Cancer Chemotherapy

A. EXPERIMENTAL MODEL (1) Intravenous Injection)

1. Method and Material

a. Animal : Fisher 344 rat, male
200-250g

b. Tumor model

Fisher Bladder Carcinoma
Tumor (2mm viable tumor fragment) was transplanted subcutaneously on the right frank by trocar

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c. Treatment was started when tumor size is about 1.5 cm.(2 weeks after implantation.)....tumor weight:1.0 ± 0.3g
The drug was administered Intravenously (through the penile vein)

Group A : 5-FU 20mg/kg (3H-5-FU30μCi) + saline
B : 5-FU 20mg/kg (3H-5-FU30μCi) + HA 15mg/kg
C : 5-FU 20mg/kg (3H-5-FU30μCi) + HA 15mg/kg + (3H-HA30μCi)

2. Sample Collection

a. accumulation of ADR, 5-FU in tumor tissue and liver

(1). Tumor was surgically removed (and blood was collected) at *predeterminated time after drug administration. Tumor weight was measured (and blood was centrifuged to obtain a plasma sample.)
* 15min, 60 min, 3hr, 4hrs,.... after drug administration.....
Liver was removed for radioactivity counting at the same time.

(2). Radioactivity level in tumor tissue was counted, using a liquid scintillation counter.

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3. Conclusion

Radioactivity in Tumor Tissue and Liver

			Tumor	Liver
15min	3H-5FU	(n=6)	2810±165	18680±625
	3H-5FU+HA	(n=6)	352±190	23593±1460
	3H-5FU+3H-HA	(n=4)	4087±681	32060±2145
60min	3H-5FU	(n=3)	1751±149	5451±841
	3H-5FU+HA	(n=4)	2599±489	8265±1849
3hrs	3H-5FU	(n=6)	1493±227	2230±449
	3H-5FU+HA	(n=6)	2512±449	2897±340
	3H-5FU+3H-HA	(n=4)	3606±929	6977±1633
5hrs	3H-FU	(n=3)	853±129	1129±70
	3H-5FU+HA	(n=3)	1981±479	1754±248
	3H-5FU+3H-HA	(n=3)	2168+163	3018+325

mean± S.E.

HA : 15 mg/kg (30μCi/kg)

5-FU : 20mg/kg (30μCi/kg)

See Figure 5' of Page 5/12 of the Figures which comprises a graph entitled "RADIOACTIVITY IN TUMOR TISSUE" comparing CPM on the vertical with time in Minutes on the horizontal (for example 100, 200, 300).

1. Radioactivity in tumor tissue in 5-FU + HA group is higher than that in 5-FU group. There is significant difference (p>0.05, ANOVA) between with and without HA at 3hrs after injection. The high intratumor concentration was retained for a prolonged time in 5-FU+HA group. (This retention was confirmed by the intratumor injection study.)
2. These results teach that HA can enhance 5-FU uptake in tumor tissue. This phenomenon results from HA distribution (in tumor tissue HA may be lost from the extracellular matrix) and the vascular uniqueness of tumor tissue (hyperpermiability of tumor vessels to macromolecular drug, HA).

As many changes can be made to the invention without departing from the scope of the invention, it is intended that all material contained herein be interpreted as illustrative of the invention and not in a limiting sense.

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THE EMBODIMENTS OF THE INVENTION IN WHICH AN EXCLUSIVE PROPERTY OR PRIVILEGE IS CLAIMED ARE AS FOLLOWS:

1. A pharmaceutical composition comprising a plurality of effective non-toxic dosage amounts of a composition for topical administration to the site of pathology and/or trauma of skin and/or exposed tissue of a human patient in need of treatment suffering from a disease or condition, each such dosage amount comprising a therapeutically effective non-toxic (to the patient) dosage amount of a drug for the treatment of the disease and/or condition of the skin and/or exposed tissue at the site of the pathology and/or trauma and an effective non-toxic dosage amount of hyaluronic acid and/or salts thereof and/or homologues, analogues, derivatives, complexes, esters, fragments, and/or sub-units of hyaluronic acid to transport (to facilitate or cause the transport of) the drug to the site of the pathology and/or trauma of the disease or condition.

2. The pharmaceutical composition of Claim 1, wherein each of the plurality of effective non-toxic dosage amounts of the composition making up the pharmaceutical composition comprises at least about 5-10mg of the form of hyaluronic acid per cm^2 of the skin and/or exposed tissue to which the composition is to be applied.

3. The pharmaceutical composition of Claim 1 or 2 wherein the hyaluronic acid and/or salts thereof and/or homologues, analogues, derivatives, complexes, esters, fragments, and/or sub-units of hyaluronic acid is hyaluronic acid and/or a salt thereof.

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4. The pharmaceutical composition of Claim 1, 2 or 3 wherein the molecular weight of the form of hyaluronic acid is less than about 750,000 daltons.

5. The pharmaceutical composition of Claim 1, 2, 3 or 4 wherein the disease and/or condition of the skin and/or exposed tissue at the site of the trauma and/or pathology is selected from at least one of basal cell carcinoma, the precancerous, often recurrent, actinic keratoses lesions, fungal lesions, "liver" spots, squamous cell tumours, metastatic cancer of the breast to the skin, primary and metastatic melanoma in the skin, malignancies and/or tumours in the skin, genital warts (condyloma acuminata), cervical cancer, and HPV (Human Papilloma Virus) including HPV of the cervix, psoriasis (both plaque-type psoriasis and nail bed psoriasis), corns on the feet and hair loss on the head of pregnant women.

6. The pharmaceutical composition of Claim 1, 2, 3, 4 or 5 wherein the drug comprises an effective non-toxic dosage amount which inhibits prostaglandin synthesis.

7. The pharmaceutical composition of Claim 6 wherein the drug is a non-steroidal anti-inflammatory drug (NSAID).

8. The pharmaceutical composition of Claim 1, 2, 3, 4 or 5 wherein the drug is an anti-cancer drug.

9. The pharmaceutical composition of Claim 8 wherein the anti-cancer drug is selected from novantrone.

10. The pharmaceutical composition of Claim 7 wherein the NSAID is selected from diclofenac, indomethacin, naproxen, and (+/-) tromethamine salt of ketorolac.

11. The pharmaceutical composition of Claim 7 wherein the NSAID is selected from IBUPROFEN, PIROXICAM, Propionic Acid derivatives, aceytylsalicylic acid and Flunixin.

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12. A topically applicable percutaneous (intracutaneous) penetrating (best targeting the epidermis) systemic independent acting (not acting essentially through the blood) pharmaceutical composition comprising a plurality of dosage amounts, each dosage amount comprising, pharmaceutical excipients suitable for topical application, a therapeutically effective (to treat and to assist to resolve a disease and/or condition of the skin and exposed tissue, selected from at least one of basal cell carcinoma, the precancerous, often recurrent, actinic keratoses lesions, fungal lesions, "liver" spots, squamous cell tumours, metastatic cancer of the breast to the skin, malignancies and/or tumours in the skin primary and metastatic melanoma in the skin, genital warts (condyloma acuminata), cervical cancer, and HPV (Human Papilloma Virus) including HPV of the cervix, psoriasis (both plaque-type psoriasis and nail bed psoriasis), corns on the feet and hair loss on the head of pregnant women, non-toxic (to the patient) dosage amount of a non-steroidal anti-inflammatory drug (NSAID) selected from diclofenac, indomethacin, naproxen, and (+/-) tromethamine salt of ketorolac and an effective non-toxic amount of hyaluronic acid and/or salts thereof to facilitate or cause the NSAID's rapid transport by the form of the hyaluronic acid to the site in the skin including the epidermis or exposed tissue of the disease or condition into the tissue to remain there for a prolonged period of time to assist to treat and assist to resolve the disease or condition by blocking prostaglandin synthesis.

13. The pharmaceutical composition of Claim 12 wherein the effective non-toxic dosage amount of the hyaluronic acid and/or salts thereof to transport the drug into the skin and/or exposed tissue exceeds about 5 mg. - 10 mg. for each 1 cm^2 of skin or exposed tissue area of the disease or condition to which the dosage amount is to be applied.

14. The pharmaceutical composition of Claim 13 wherein the molecular weight of the hyaluronic acid and/or salts is less than about 750,000 daltons.

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15. A pharmaceutical composition from which dosage amounts may be taken and applied to the skin to treat a disease or condition in humans, the pharmaceutical composition comprising:

(1) a medicinal and/or therapeutic agent suitable for treating a disease or condition in the skin and/or exposed tissue in humans, and

(2) hyaluronic acid and/or salts thereof and/or homologues, analogues, derivatives, complexes, esters, fragments, and sub-units of hyaluronic acid, in a form suitable for administration to the skin and/or exposed tissue in humans; characterized in that an effective dosage amount comprising effective non-toxic dosage amounts of components (1) and (2) taken and administered from said composition (i) is available in the skin and/or exposed tissue upon administration to treat said disease or condition in humans by penetration at the site to be treated to the site of trauma and/or pathology, and (ii) comprises an effective non-toxic dosage amount of component (2) to transport (facilitate or cause the transport of) component (1) immediately upon administration percutaneously into the skin including the epidermis, to the site to be treated where it remains a prolonged time, accumulating there and from which it is discharged via the lymphatic system.

16. The pharmaceutical composition of Claim 15 wherein the effective non-toxic dosage amount of component (2) comprises an effective non-toxic dosage amount of at least about 5-10mg/cm^2 of the skin and/or exposed tissue to which the dosage amounts taken from the pharmaceutical composition is to be applied.

17. The pharmaceutical composition of Claim 15 or 16, wherein the hyaluronic acid and/or salts thereof and/or homologues, analogues, derivatives, complexes, esters, fragments, and/or sub-units of hyaluronic acid is hyaluronic acid and/or a salt thereof.

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18. The pharmaceutical composition of Claim 15, 16 or 17, wherein the molecular weight of the form of hyaluronic acid is less than about 750,000 daltons.

19. The pharmaceutical composition of Claim 15, 16, 17 or 18 wherein the medicinal and/or therapeutic agent is a non-steroidal anti-inflammatory drug (NSAID).

20. A pharmaceutical composition comprising:

(1) a medicinal and/or therapeutic agent in a therapeutically effective amount to treat disease or condition of the skin and/or exposed tissue;

and (2) hyaluronic acid and/or salts thereof and/or homologues, analogues, derivatives, complexes, esters, fragments, and subunits of hyaluronic acid,

characterized in that said composition

(a) is in a dosage form which is suitable for administration to skin and/or exposed tissue;

and (b) is in such an amount and in such form that (i) component (1) is in an effective dosage amount to treat said disease or condition by penetration at the site of the skin or exposed tissue to be treated, and (ii) component (2) is immediately available to transport (facilitate or cause the transport of) component (1) to the site of trauma and/or pathology of the disease or condition to be treated, percutaneously into the skin or exposed tissue where the composition resides and accumulates for a prolonged period, and which component (2) is in an effective non-toxic dosage amount to transport (facilitate or cause the transport of) component (1) upon administration, percutaneously into the skin or exposed tissue to the site of the trauma and/or pathology.

21. The composition of Claim 20 wherein the form of hyaluronic acid in the composition comprises hyaluronic acid and/or salts thereof.

22. The composition of Claim 20 or 21 wherein the effective amount of the form of hyaluronic acid in the

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composition exceeds about 5-10 mg per square centimeter (cm^2) of skin or exposed tissue to which it is to be applied.

23. The composition of Claim 20, 21 or 22 wherein the molecular weight of the form of hyaluronic acid is less than about 750,000 daltons.

24. The composition of Claim 20, 21, 22 or 23 wherein the drug is a non-steroidal anti-inflammatory (NSAID).

25. The composition of Claim 20, 21, 22 or 23 wherein the drug is an anti-cancer agent.

26. The use of:

(1) a medicinal and/or therapeutic agent,

and (2) hyaluronic acid and/or salts thereof and/or homologues, analogues, derivatives, complexes, esters, fragments, and subunits of hyaluronic acid,

in the manufacture of a pharmaceutical composition for treating a disease or a condition of the skin and/or exposed tissue in a therapy wherein dosage amounts taken from the composition each comprise:

(1) a therapeutically effective amount of said medicinal and/or therapeutic agent and

(2) a therapeutically effective amount of the hyaluronic acid and/or salts thereof and/or homologues, analogues, derivatives, complexes, esters, fragments, and sub-units of hyaluronic acid, the pharmaceutical composition being characterized in that for each dosage amount taken from the pharmaceutical composition, the amount of component (2) is immediately available to transport component (1) percutaneously to the site of trauma and/or pathology caused by a disease and/or condition of the skin and/or exposed tissue into the epidermis of the skin and/or exposed tissue to be treated, and component (2) is in an effective non-toxic amount to transport (facilitate or cause the transport of) component (1) into the skin or exposed tissue (for example into the epidermis) to the site of trauma and/or pathology.

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27. The use of Claim 26 wherein component (2) is hyaluronic acid and/or salts thereof having a molecular weight less than about 750,000 daltons.

28. The use of Claim 26 or 27 wherein the dosage amount of component (2) in the amount of the composition taken from the composition (to be taken from the composition) and applied to the skin and/or exposed tissue is present in a dose amount greater than about 5-10 mg per cm^2 of skin or exposed tissue to which the dosage amount is to be applied.

29. The use of Claim 26, 27 or 28 wherein component (1) is a non-steroidal anti-inflammatory drug (NSAID).

30. The use of:

(1) a medicinal and/or therapeutic agent which inhibits prostaglandin synthesis,

and (2) hyaluronic acid and/or salts thereof,

in the manufacture of a pharmaceutical composition for treating disease or condition at the site of trauma and/or pathology of the skin and exposed tissue in a therapy wherein a dosage amount comprises a therapeutically effective amount of said medicinal and/or therapeutic agent and a therapeutically effective amount of the hyaluronic acid and/or salts thereof having a molecular weight less than about 750,000 daltons, the use being characterized in that the amount of component (2) is immediately available to transport component (1) percutaneously into the epidermis at the site of the skin or exposed tissue to be treated and component (2) is an effective non-toxic amount to transport (facilitate the transport) of component (1) into the skin or exposed tissue.

31. The use of Claim 30 wherein the dosage amount of component (2) is present in a dose greater than 5-10 mg per cm^2 of skin or exposed tissue to which the composition is to be applied.

32. The pharmaceutical composition of Claim 20, 21, 22, 23, 24, or 25 include pharmaceutically compatible excipients

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to provide a form for ease of administration to the skin or exposed tissue for transport into the epidermis.

33. The pharmaceutical composition of Claim 32 comprising a gel squeezed from a tube as a ribbon of gel and which dosage amount is in the form of the ribbon of gel "X" cm long containing the effective non-toxic amounts of the drug and form of hyaluronic acid.

34. The pharmaceutical composition of Claim 32 comprising a cream taken from a jar to be scooped from the jar in a dosage amount comprising the effective amount of drug and effective amount of the form of hyaluronic acid.

35. The pharmaceutical composition of Claim 32 comprising a form selected from a gel, lotion or cream.

36. A method of treating a disease or condition of the skin or exposed tissue comprising administering topically a non-toxic dosage amount of a composition comprising pharmaceutical excipients suitable for topical application, a therapeutically effective (to treat and to assist to resolve the disease or condition) non-toxic dosage amount of a drug which inhibits prostaglandin synthesis, and an effective non-toxic dosage amount of hyaluronic acid and/or salts thereof and/or homologues, analogues, derivatives, complexes, esters, fragments, and/or sub-units of hyaluronic acid to transport (facilitate the transport of) the drug into the skin or exposed tissue at the site of the disease or condition to be treated percutaneously into epidermis and rubbing the composition into the skin and/or exposed tissue.

37. The method of Claim 36 wherein the form of hyaluronic acid is hyaluronic acid and salts thereof.

38. The method of Claim 36 or 37 wherein the amount of the hyaluronic acid exceeds about 5mg./cm^2 of the skin or exposed tissue to which the dosage amount of the composition is applied.

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39. The method of Claim 36, 37 or 38 wherein the molecular weight of the form of hyaluronic acid is less than about 750,000 daltons.

40. The method of Claim 36, 37, 38 or 39 wherein the treatment is applied daily for a number of weeks.

41. The method of Claim 36, 37, 38, 39 or 40 wherein the drug is a non-steroidal anti-inflammatory drug (NSAID).

42. The method of Claim 41 wherein the NSAID is selected from diclofenac, indomethacin, naproxen, and (+/-) tromethamine salt of ketorolac.

43. The method of Claim 41 wherein the NSAID is selected from IBUPROFEN, PIROXICAM, Propionic Acid derivatives, aceytylsalicylic acid and Flunixin.

44. The method of treatment of Claim 41, 42 or 43 wherein the treatment comprises applying effective dosage amounts of the composition, a number of times daily for a period of weeks to clear the trauma or pathology.

45. A container containing compositions for topical application to the skin and/or exposed tissue of a human comprising a plurality of dosage amounts of each of (a) a drug and (b) hyaluronic acid and/or sodium hyaluronate, each dosage amount comprising an effective non-toxic dosage amount of the drug to treat a disease and/or condition of the skin and an effective non-toxic dosage amount of hyaluronic acid and/or sodium hyaluronate to transport the drug into the skin and/or exposed tissue to the site of the pathology and/or trauma.

46. The container of Claim 45 wherein the hyaluronic acid and/or sodium hyaluronate has a molecular weight less than about 750,000 daltons to transport the drug into the skin and/or exposed tissue.

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47. The container of Claim 45 wherein the effective dosage amount of the hyaluronic acid and/or sodium hyaluronate is in a dosage amount exceeding 5mg/cm^2 of the hyaluronic acid and/or sodium hyaluronate in the dosage amount discharged.

48. The container of Claim 45, 46 or 47 wherein means are provided to assist the removal from the container of an effective dosage amount of the composition in the container for use to apply to the skin or exposed tissue at the site of trauma and/or pathology to treat the disease and/or condition.

49. The container of Claim 48 wherein the container is a tube and said means comprises a mouth opening of the tube to assist in the discharge of an effective dosage amount for discharge from the tube.

50. Percutaneous (intercutanous) delivery of a therapeutically effective dosage amount of a drug and which inhibits prostaglandin synthesis in a pharmaceutical composition the drug being transported to the site of, on, or in the skin and/or exposed tissue of a human of trauma and/or pathology to treat a disease or condition of the skin and/or exposed tissue, the delivery comprising topically administering to the skin and/or exposed tissue site of the trauma and/or pathology, the therapeutically effective, non-toxic (to the patient) dosage amount of the drug which inhibits prostaglandin synthesis, in a composition which comprises an effective non-toxic amount of hyaluronic acid and/or salts thereof sufficient to transport, (facilitate or cause the transport of), the drug to the epidermis to the site of the trauma and/or pathology to block the synthesis of prostaglandins.

51. The percutaneous delivery of Claim 50 wherein the amount of hyaluronic acid and/or salts thereof exceeds at least about 5mg/cm^2 of the skin and/or exposed tissue to which the composition is to be applied.

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52. The percutaneous delivery of Claim 50 or 51 wherein the molecular weight of the hyaluronic acid and/or salts is less than about 750,000 daltons.

53. The percutaneous delivery of Claim 50, 51 or 52 wherein the drug comprises an anti-cancer drug for administration to a tumour or malignancy in the skin and/or exposed tissue.

54. The percutaneous delivery of Claim 53 wherein the drug is 10 mg of novantrone in the dosage amount of the composition and the hyaluronic acid and/or salts thereof is in excess of about 5 mg of sodium hyaluronic per cm^2 of the skin or exposed tissue, about 2.5% of the composition, for the percutaneous transport of the novantrone.

55. Use of a pharmaceutical composition to treat a disease or condition by the application of the composition to the skin and/or exposed tissue of a human, the amount of the composition, administered comprising together with pharmaceutical excipients suitable for topical application, a therapeutically effective non-toxic (to a human) amount of a drug which inhibits prostaglandin synthesis and an effective non-toxic dosage amount of at least about 5mg/cm^2 of skin and/or exposed tissue of hyaluronic acid and/or salts thereof having a molecular weight less than about 750,000 daltons effective to transport the drug (to facilitate or cause the transport of the drug) percutaneously into the skin especially the epidermis at the site of the disease or condition to be treated, thereby blocking prostaglandin synthesis to enable the macrophages (and N.K. cells) to resolve the disease or condition.

56. A method of abating pain in skin and exposed tissue of a human suffering from a disease or condition comprising administering a composition comprising an effective non-toxic dosage amount of a drug which relieves pain and an effective non-toxic dosage amount of the hyaluronic acid and/or salts thereof and/or homologues, analogues, derivatives, complexes,

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esters, fragments, and/or subunits of hyaluronic acid in an amount exceeding 10-20 mg. per square cm (cm^2) of the skin or exposed tissue to which it is applied for percutaneous transport of the drug by the form of hyaluronic acid into the epidermis proximate the paccinian nerve bundles to give pain relief.

57. The method of abating pain of Claim 56 wherein the form of hyaluronic acid is hyaluronic acid and/or salts thereof.

58. The method of abating pain of Claim 56 or 57 wherein the drug is an NSAID.

59. The method of Claim 56, 57 or 58 wherein the molecular weight of the form of hyaluronic acid is less than about 750,000 daltons.

60. A composition from which dosage amounts may be taken and applied to human skin and/or exposed tissue suffering pain for abating the pain, the composition comprising a plurality of dosage amounts which may be taken and applied, each dosage amount comprising an effective non-toxic dosage amount of an NSAID and an effective non-toxic dosage amount of the hyaluronic acid and/or salts thereof and/or homologues, analogues, derivatives, complexes, esters, fragments, and/or subunits of hyaluronic acid exceeding 10-20 mg. per square cm (cm^2) of skin and/or exposed tissue to which it is applied, for percutaneous transport of the NSAID by the form of hyaluronic acid into the epidermis proximate the paccinian nerve bundles (superficial nerve bundles at the end of the nerves) to abate the pain.

61. The composition of Claim 60 wherein the form of hyaluronic acid is hyaluronic acid is hyaluronic acid and/or salts thereof.

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62. The composition of Claim 60 or 61 wherein the molecular weight of the form of hyaluronic acid is less than about 750,000 daltons.

63. The composition of Claim 60, 61, or 62 in a container including means for assisting the discharge of an effective dosage amount from the container.

64. The composition of Claim 63 wherein the container is a tube and said means comprises a mouth opening of predetermined diameter through which the effective dosage amount of the composition is discharged.

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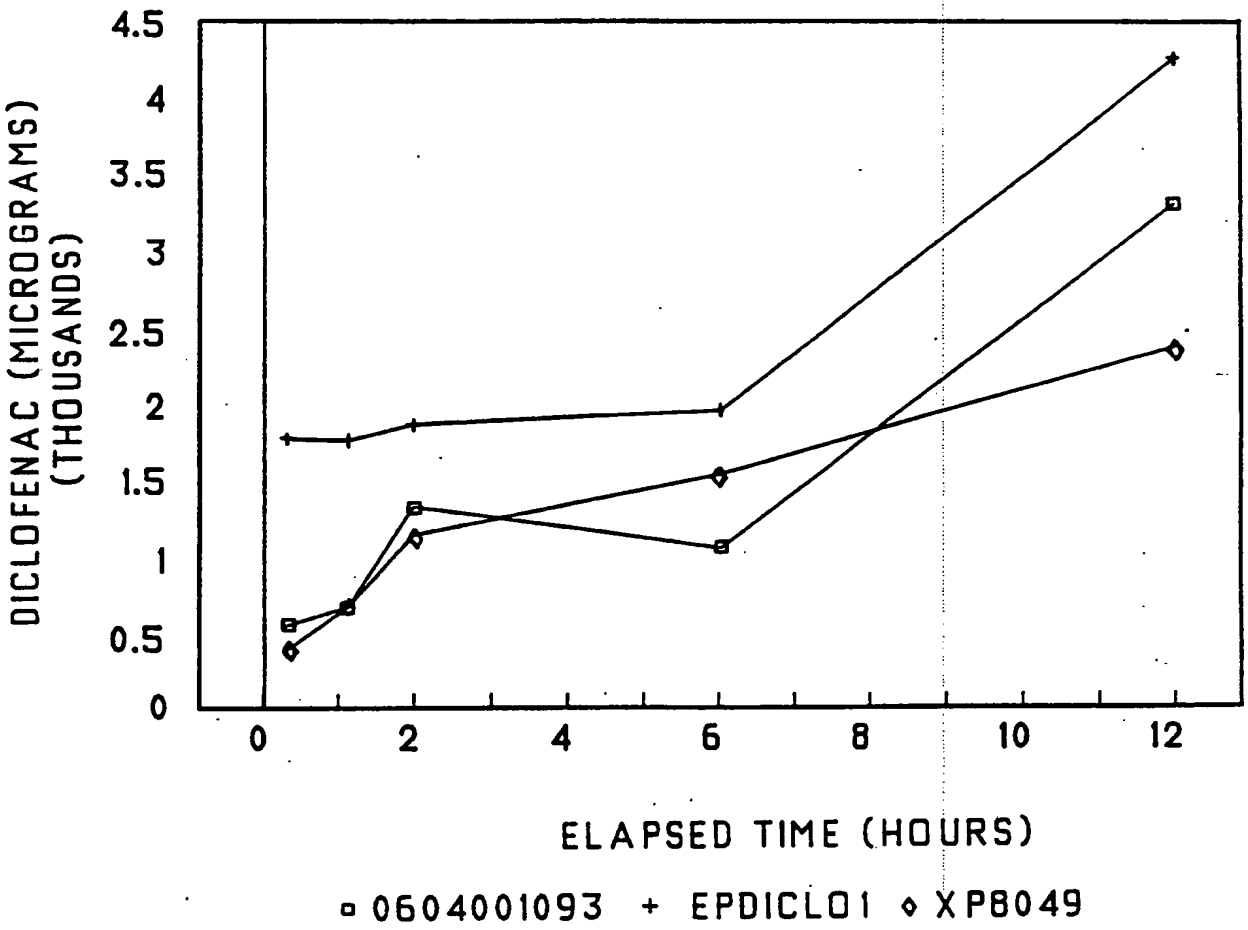

FIG.1.

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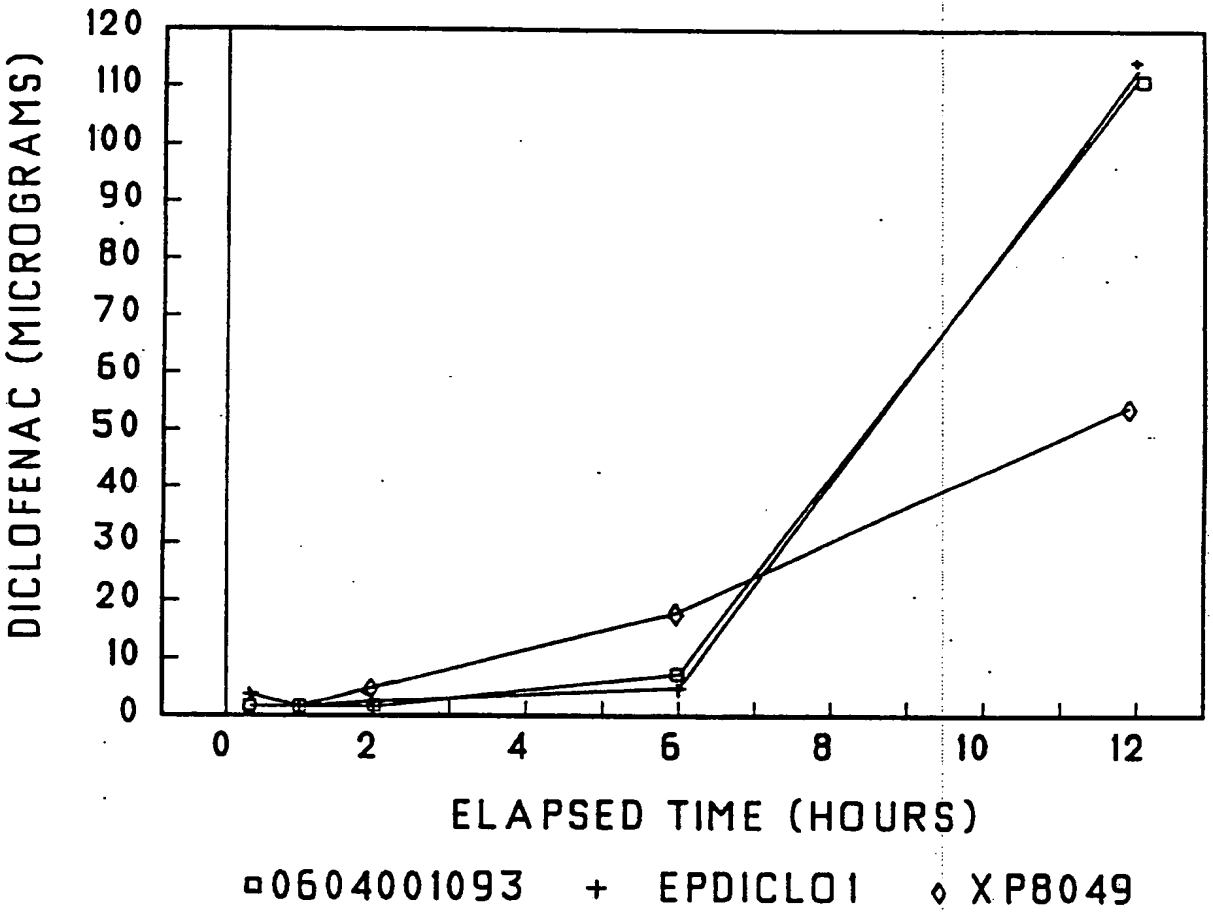

FIG 2'

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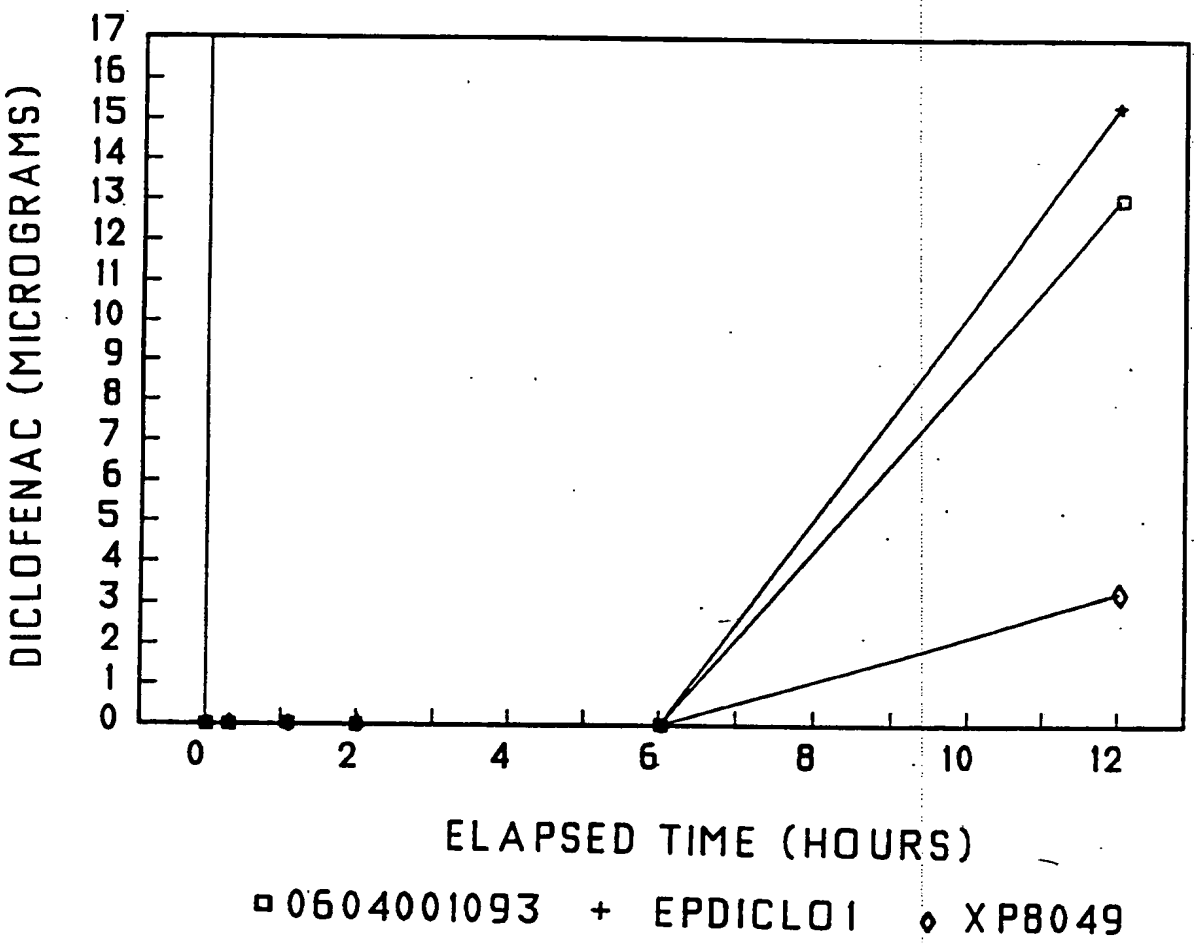

FIG.3.

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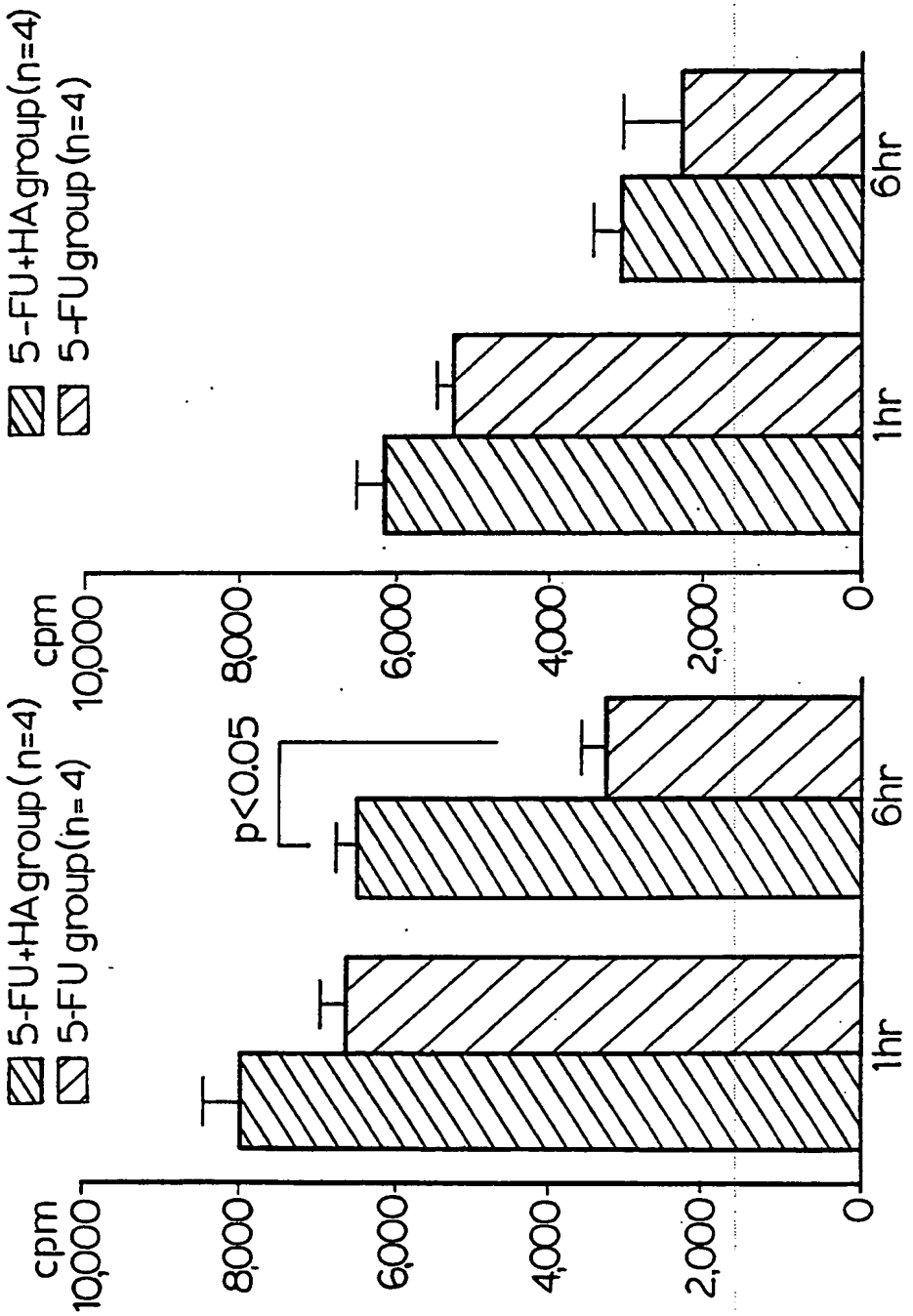

FIG 4'

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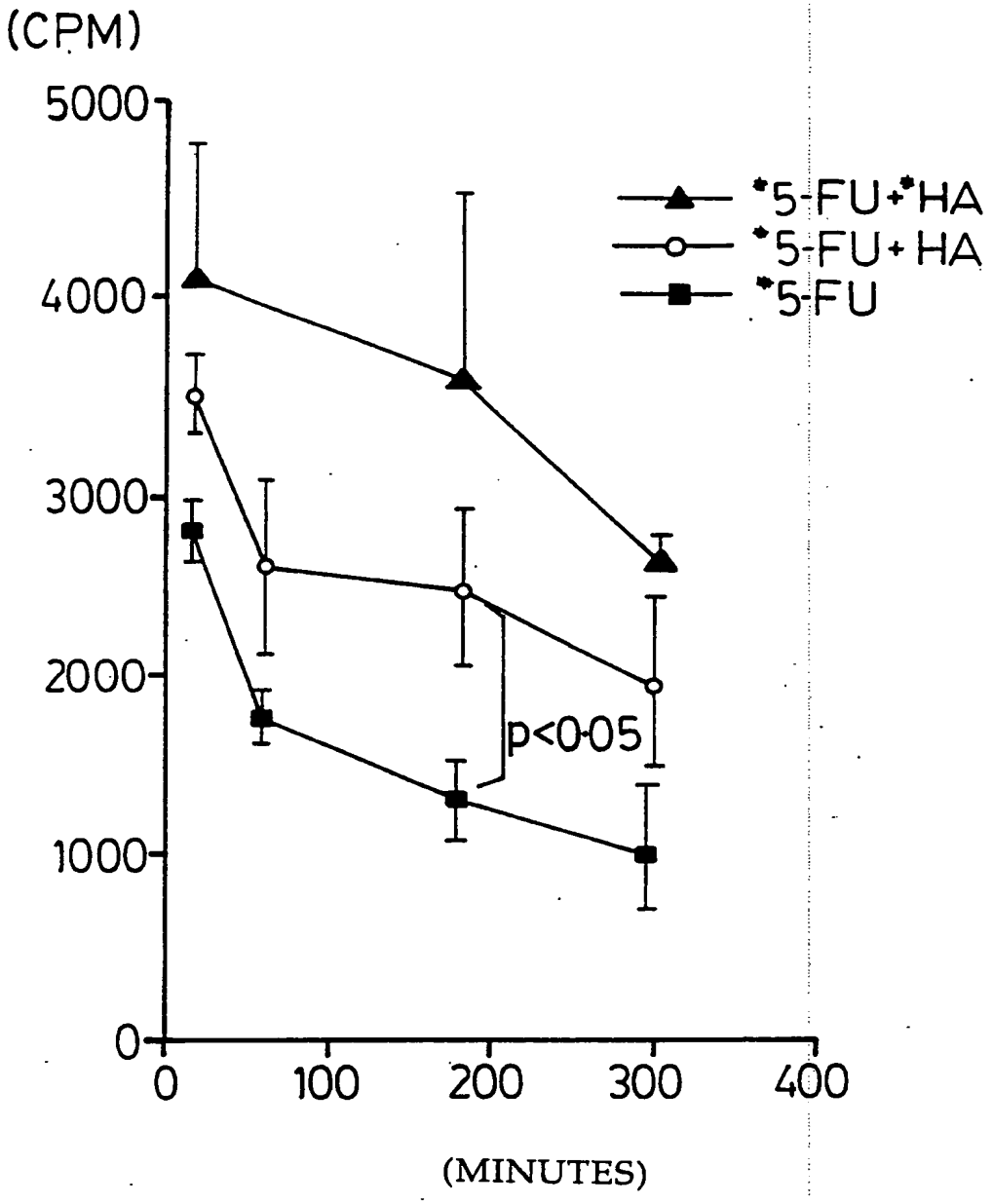

FIG. 5'

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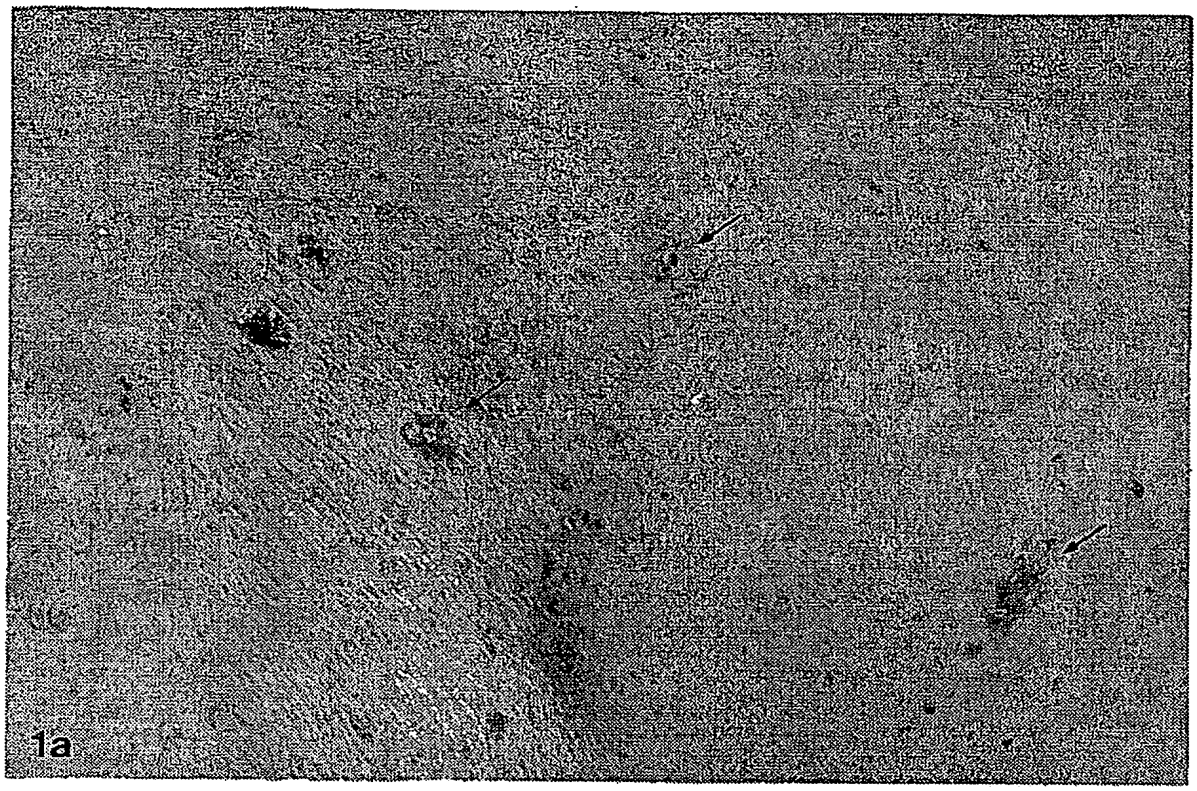

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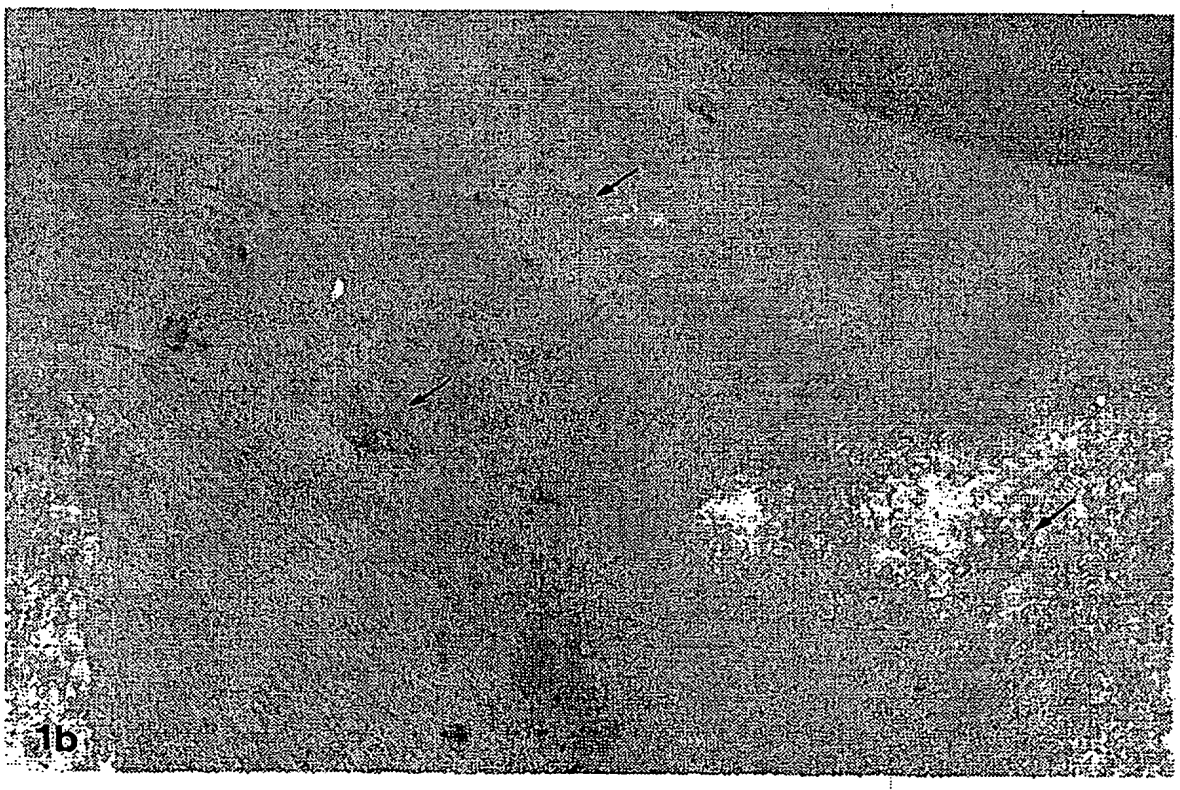

1b

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2a

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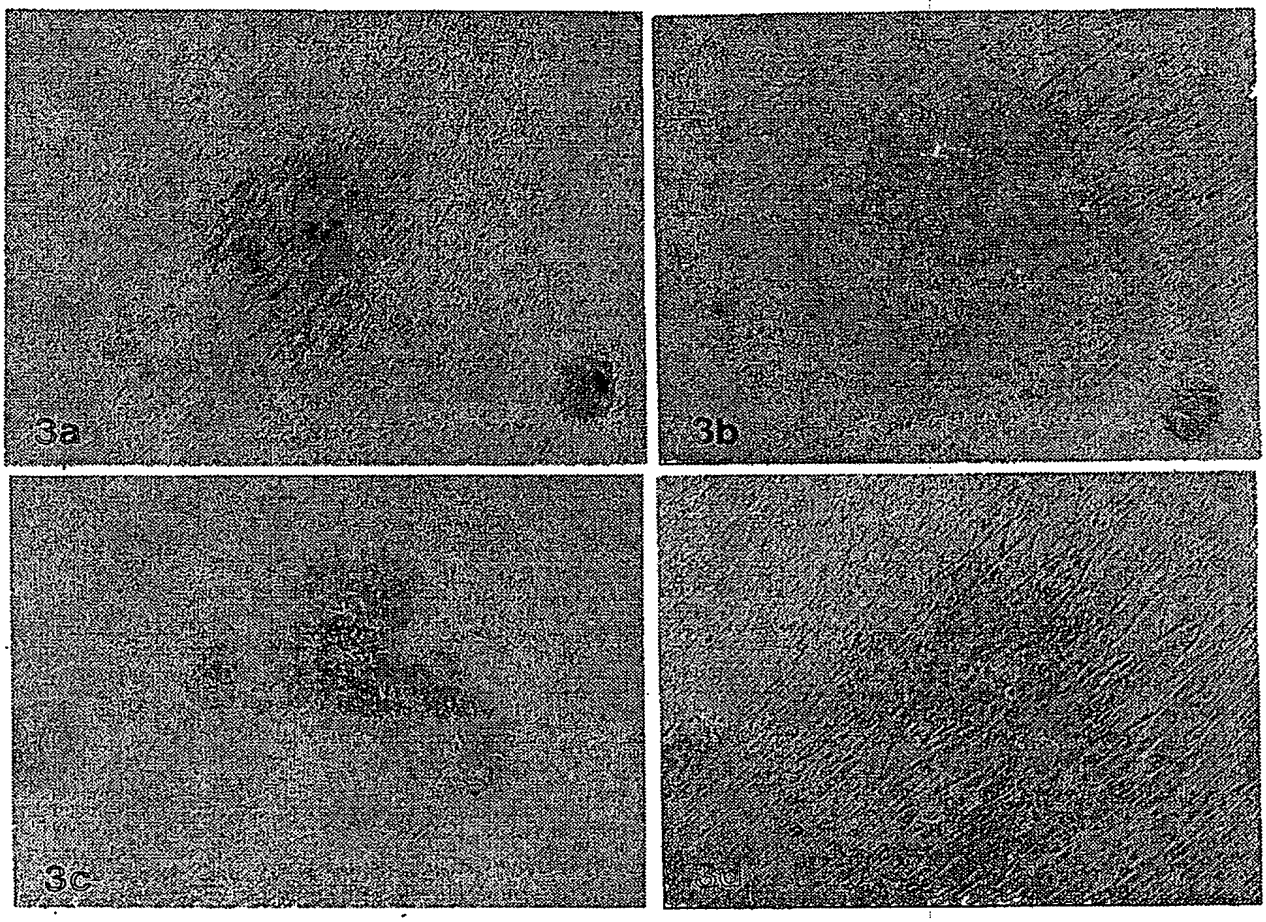

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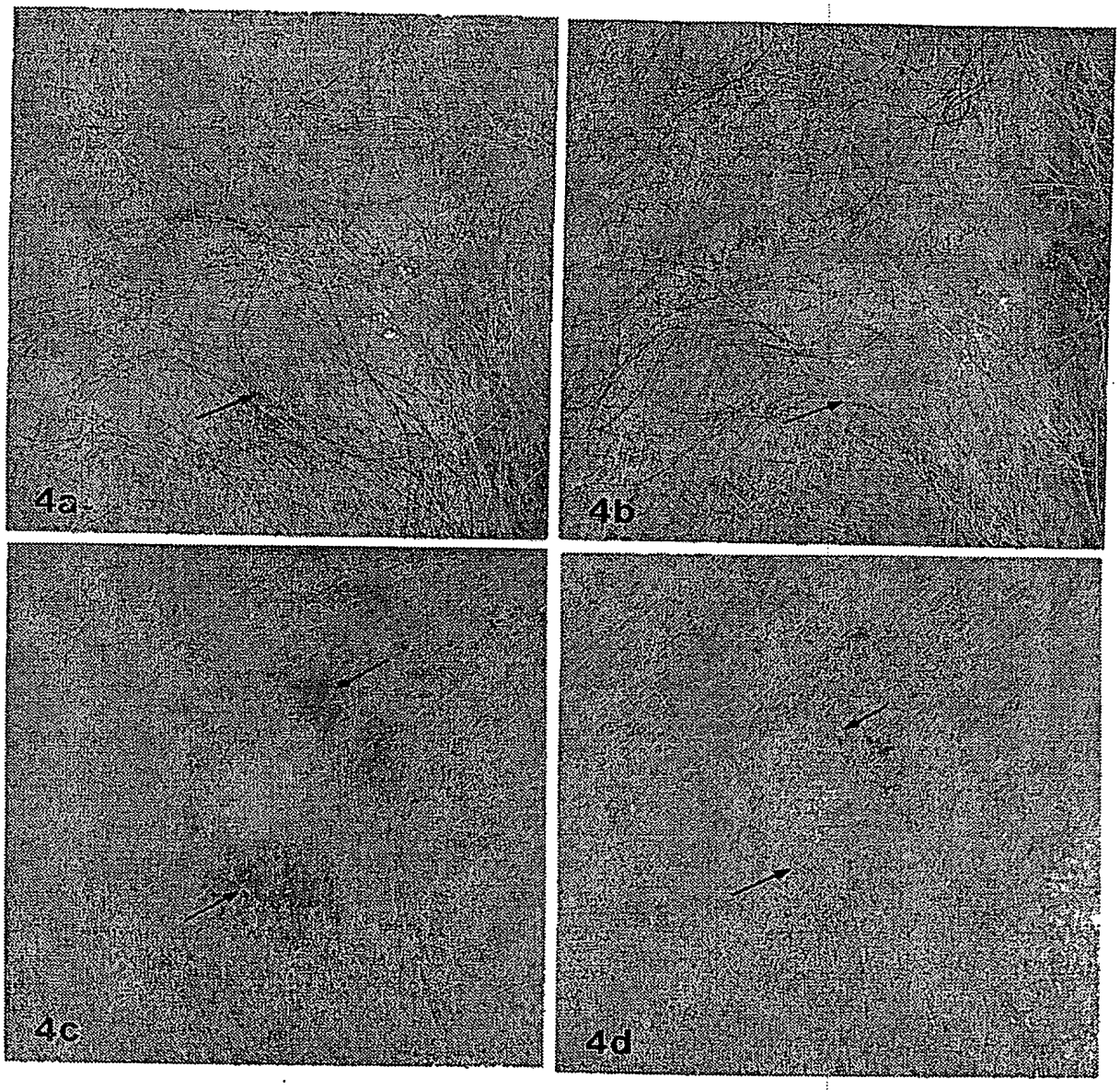

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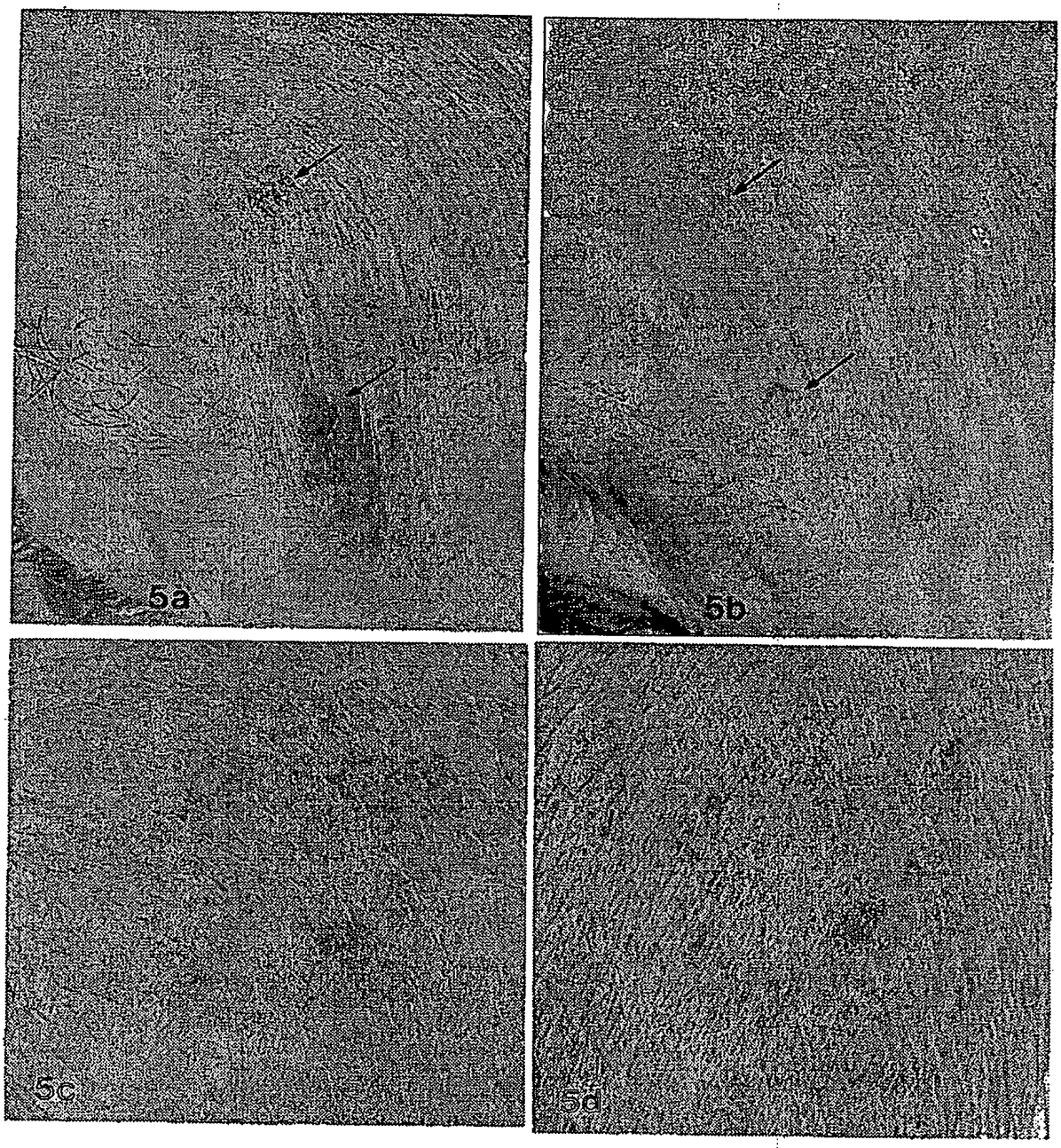

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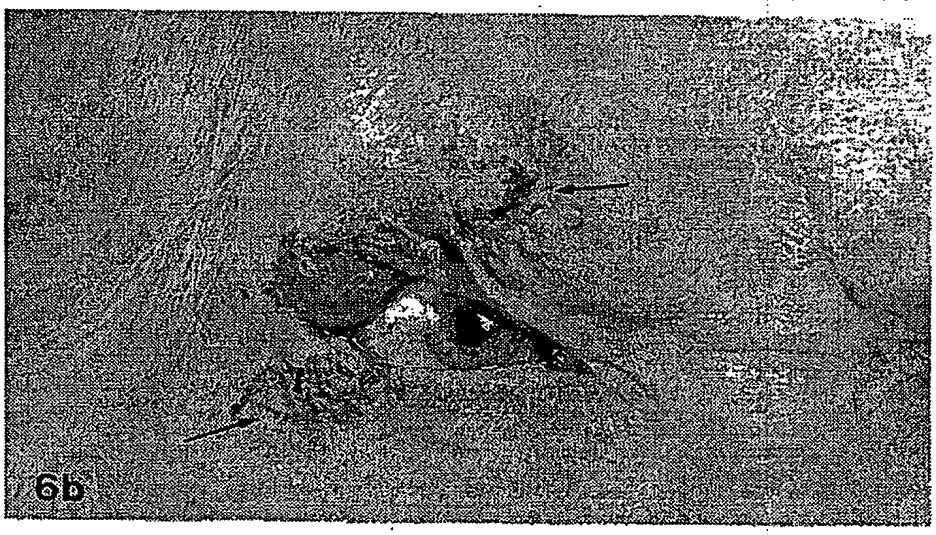
6a



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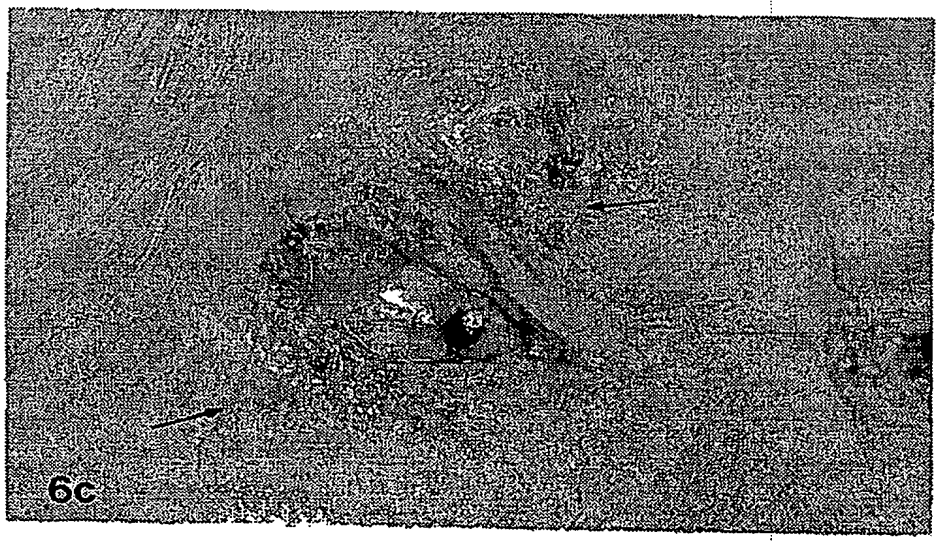

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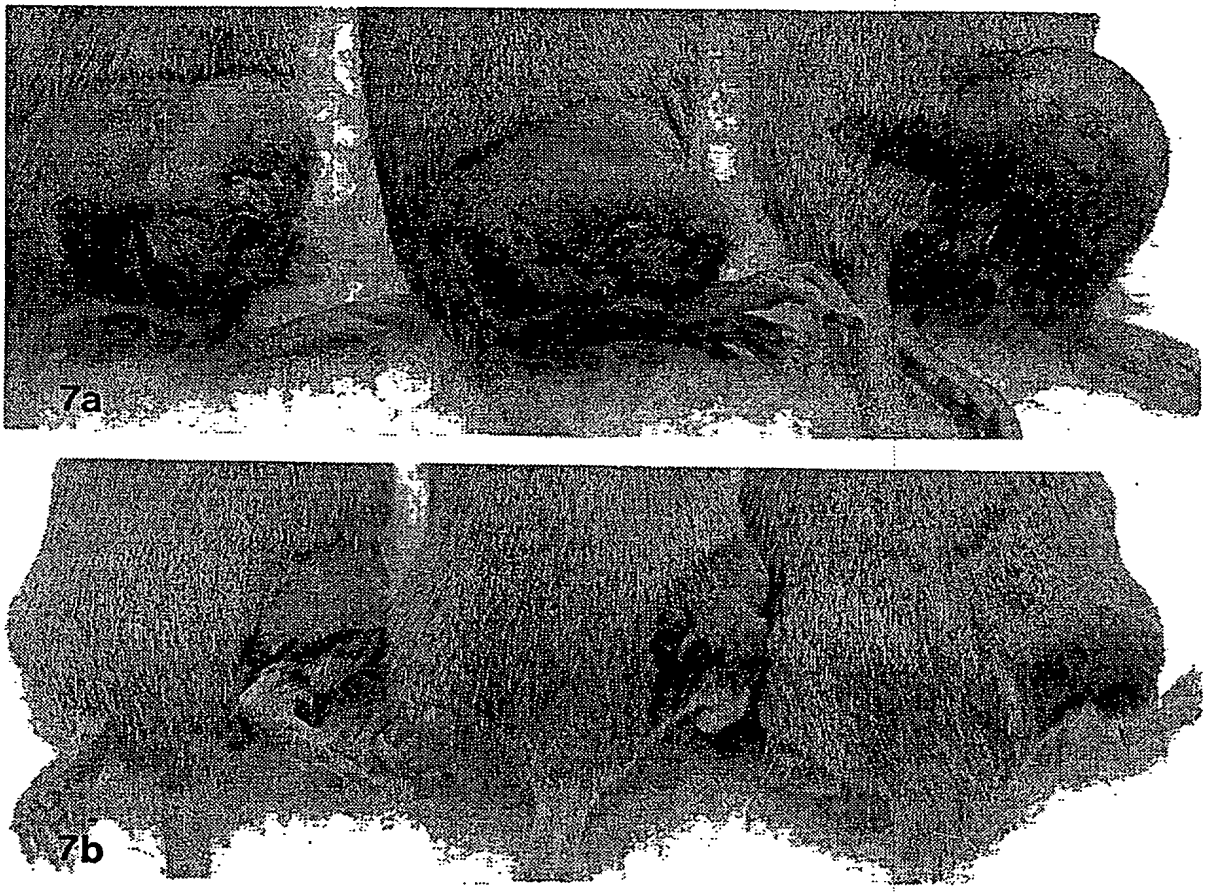

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INTERNATIONAL SEARCH REPORT

International Application No PCT/CA 93/00062

I. CLASSIFICATION OF SUBJECT MATTER (if several classification symbols apply, indicate all)[6]

According to International Patent Classification (IPC) or to both National Classification and IPC

Int.Cl. 5 A61K47/36

II. FIELDS SEARCHED

Minimum Documentation Searched[7]

Classification System	Classification Symbols
Int.Cl. 5	A61K

Documentation Searched other than Minimum Documentation to the Extent that such Documents are Included in the Fields Searched[8]

III. DOCUMENTS CONSIDERED TO BE RELEVANT[9]

Category °	Citation of Document, [11] with indication, where appropriate, of the relevant passages [12]	Relevant to Claim No.[13]
X	WO,A,9 104 058 (NORPHARMCO) 4 April 1991 see the whole document ---	1-64
X	EP,A,0 197 718 (FIDIA SPA) 15 October 1986 cited in the application see claims 1-6,9,10,14,15 see page 4, line 30 - page 5, line 1 see page 7, line 30 - page 9, line 15 see page 11, paragraph 3 see page 12, line 11 - line 18 see page 19, line 17 - line 22 --- -/--	1-8, 10-53, 55-64

° Special categories of cited documents :[10]

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"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

IV. CERTIFICATION

Date of the Actual Completion of the International Search	Date of Mailing of this International Search Report
27 APRIL 1993	[illegible]
International Searching Authority	**Signature of Authorized Officer**
EUROPEAN PATENT OFFICE	VENTURA AMAT A.

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III. DOCUMENTS CONSIDERED TO BE RELEVANT (CONTINUED FROM THE SECOND SHEET)

Category °	Citation of Document, with indication, where appropriate, of the relevant passages	Relevant to Claim No.
X	US,A,4 937 254 (WARREN D. SHEFFIELD ET AL.) 26 June 1990 cited in the application see claims 1,2,5 see column 3, line 8 - line 20 see column 3, line 39 - line 41 see column 3, line 66 - column 4, line 5 see column 4, line 11 - line 29	1-3,5-7, 10,11
X	EP,A,0 368 253 (UNION CARBIDE CHEMICALS AND PLASTICS COMPANY INC.) 16 May 1990 see claims 1,4,6 see column 8, line 55 - column 9, line 10 see column 9, line 44 - line 51	1-3,5-7, 10,11

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ANNEX TO THE INTERNATIONAL SEARCH REPORT ON INTERNATIONAL PATENT APPLICATION NO.

CA 9300062
SA 70222

This annex lists the patent family members relating to the patent documents cited in the above-mentioned international search report.
The members are as contained in the European Patent Office EDP file on
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Patent document cited in search report	Publication date	Patent family member(s)		Publication date
WO-A-9104058	04-04-91	AU-A-	6433090	18-04-91
		CN-A-	1051503	22-05-91
		EP-A-	0445255	11-09-91
		JP-T-	4504579	13-08-92
EP-A-0197718	15-10-86	AU-B-	592077	04-01-90
		BE-A-	904547	03-10-86
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		JP-A-	2196728	03-08-90

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INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

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(81) Designated States: AT, AU, BB, BG, BR, CA, CH, CZ, DE, DK, ES, FI, GB, HU, JP, KP, KR, KZ, LK, LU, MG, MN, MW, NL, NO, NZ, PL, PT, RO, RU, SD, SE, SK, UA, US, European patent (AT, BE, CH, DE, DK, ES, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, ML, MR, NE, SN, TD, TG).

Published
With international search report.

(54) Title: GRAFT COPOLYMERS OF POLYCATIONIC SPECIES AND WATER-SOLUBLE POLYMERS, AND USES THEREOF

(57) Abstract

In accordance with the present invention, there are provided methods to render cells non-adhesive and/or non-immunogenic with respect to macromolecules typically encountered in culture media or in physiological media.

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GRAFT COPOLYMERS OF POLYCATIONIC SPECIES AND WATER-SOLUBLE POLYMERS, AND USES THEREOF

The present invention relates to methods for rendering cells non-adhesive. In another aspect, the present invention relates to methods for rendering cells non-immunogenic. In yet another aspect, the present invention relates to methods for the stabilization of liposomes. In a further aspect, the present invention relates to methods for the *in vitro* generation of neural networks.

BACKGROUND OF THE INVENTION

Water-soluble polymers, such as polyethylene glycols (PEGs), have been investigated extensively in recent years for use as biocompatible, protein repulsive, noninflammatory, and nonimmunogenic modifiers for drugs, proteins, enzymes, and surfaces of implanted materials. These characteristics have variously been attributed to a combination of properties of such polymers, e.g., nonionic character, water solubility, backbone flexibility, and volume exclusion effect in solution or when immobilized on a surface.

While extensive efforts have been made to render foreign substances, such as drugs, proteins, and the like, non-immunogenic employing water-soluble polymers such as PEG, the use of such polymers to render an individual cell non-immunogenic has not been considered in the art. If such polymers could be attached directly to a cell surface, then it is possible, due to the exclusion of proteins and macromolecules from the vicinity of the cell surface, that the cell as a whole may be rendered non-immunogenic. The ability to accomplish such attachment would be invaluable for a variety of treatment protocols.

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It is known that mammalian cell membranes have a variety of negatively charged species on their exterior. For example, negatively charged proteoglycans (PGA), glycosaminoglycans (GAG), such as chondroitin sulfate and heparin sulfate, and negatively charged lipids have all been identified on cell exteriors. Not surprisingly, polycation species such as polylysine and polyornithine interact with negatively charged cell surfaces to form a strong ionic linkage. Unfortunately, polycations (such as polylysine and polyornithine) are known to be cytotoxic, even at fairly low concentrations. Polylysine, for example, has been used as a cell fixative, and has been shown to cause cell aggregation (e.g., with human platelets).

While water-soluble polymers, having found use in a variety of biological applications, would be ideal for use in treating cells to render them non-immunogenic, their generally non-ionic nature renders them substantially unable to bind to cell membranes. Thus, for example, treatment of cells with unmodified PEG was unable to confer a non-adhesive or protein repellant character on such cells.

BRIEF DESCRIPTION OF THE INVENTION

In accordance with the present invention, we have developed methods to render cells non-adhesive and/or non-immunogenic with respect to macromolecules typically encountered in culture media or in physiological media.

The methods of the present invention can be used for a wide variety of purposes, e.g., for the treatment of cells used for implantation (thereby avoiding the need for immunosuppressive therapy), for the preservation of organs outside the body while awaiting transplant, for modifying surfaces of materials which are to be exposed to various

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components of physiological media, for the stabilization of liposomes (and prevention of their uptake by the reticuloendothelial system), and the like.

DETAILED DESCRIPTION OF THE INVENTION

In accordance with the present invention, there is provided a method to render cells non-adhesive, said method comprising contacting said cells with an effective amount of a composition comprising a polycationic species having water-soluble polymer chains grafted thereon.

As employed herein, reference to rendering cells "non-adhesive" means, in an *in vitro* setting, that cells do not stick to wells (e.g., glass, plastic, and the like), or other surfaces with which they come in contact. Instead, non-adhesive cells, as contemplated herein, spread and grow, yet remain in suspension. In an *in vivo* setting, "non-adhesive" refers to cells which do not adhere to biologically-encountered macromolecules or proteinaceous matrix (e.g., collagen matrix). As used herein, "non-adhesive" also refers to cells which have been rendered non-immunogenic, i.e., cells which are substantially non-susceptible to an immune response mediated by biological macromolecules.

"Contacting" of cells or tissues with graft copolymer compositions contemplated for use in the practice of the present invention is typically carried out *in vitro* at room temperature for a time in the range of about 0.01 up to 1 hour or longer in suitable physiological buffer (i.e., pH in the range of about 7.2-7.4; osmolarity of about 290 mOsm/kg) containing a concentration of at least about 0.005% of graft copolymer, with respect to the concentration of the polycationic species used for the preparation of the cell surface (e.g., polylysine). It is presently preferred to treat cells with a solution of graft

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copolymer containing a concentration of graft copolymer in the range of about 0.05 up to 1.0%, with concentrations of graft copolymer in the range of about 0.1 up to 0.5% being especially preferred. Those of skill in the art recognize that as the molecular weight of the polycationic species is increased, a lower concentration (determined on the same basis as set forth above) of the graft copolymer is required to produce the same beneficial effect.

As employed herein, an "effective amount" of graft copolymer compositions contemplated for use in the practice of the present invention is an amount sufficient to render said cells non-adhesive to biological macromolecules, while leaving the cells viable (as determined, for example, by suitable staining techniques). In the case of specialized cells, such as islets, it is desirable for the treated cells to retain their unique function as well as viability (i.e., the ability of islets to respond to exposure to glucose by secretion of insulin). Typically, an excess of graft copolymer is used with respect to the negative charges present on the surface of the cells to be treated. The quantity of graft copolymer required will vary depending on the cell type being treated and the concentration of cells to be treated. Typically, in the range of about 10^2-10^8 cells/ml will be treated. For example, up to about 10^8 bacterial cells/ml, up to about 100,000 fibroblasts/ml, or up to about 50,000 islets/ml will be treated.

Copolymer compositions contemplated for use in the practice of the present invention comprise a polycationic species having water-soluble polymer chains grafted thereon. Polycationic species contemplated for use in the practice of the present invention are polycationic species having sufficient charge density to allow binding of the above-described graft copolymer to cells, and include:

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polymers containing primary amine groups, secondary amine groups, tertiary amine groups or pyridinyl nitrogen(s), such as polyethyleneimine, polyallylamine, polyetheramine, polyvinylpyridine, and the like,

polysaccharides having a positively charged functionality thereon (e.g., chitosan),

polyamino acids, such as:
poly-L-histidine, poly-im-benzyl-L-histidine,
poly-D-lysine, poly-DL-lysine, poly-L-lysine,
poly-ϵ-CBZ-D-lysine, poly-ϵ-CBZ-DL-lysine,
poly-ϵ-CBZ-L-lysine,

poly-DL-ornithine, poly-L-ornithine,
poly-δ-CBZ-DL-ornithine,

poly-L-arginine,

poly-DL-alanine-poly-L-lysine;

poly(-L-histidine, L-glutamic acid)-poly-DL-alanine-poly-L-lysine;

poly(L-phenylalanine, L-glutamic acid)-poly-DL-alanine-poly-L-lysine;

poly(L-tyrosine, L-glutamic acid)-poly-DL-alanine-poly-L-lysine;

random copolymers of:
L-arginine with tryptophan, tyrosine, or serine;
D-glutamic acid with D-lysine;
L-glutamic acid with lysine, ornithine, or mixtures thereof;

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and the like, as well as mixtures of any two or more thereof.

Presently preferred polycations for use in the practice of the present invention include polylysine (i.e., poly-D-lysine (PDL), poly-DL-lysine, poly-L-lysine (PLL), poly-ε-CBZ-D-lysine, poly-ε-CBZ-DL-lysine, or poly-ε-CBZ-L-lysine), polyornithine (i.e., poly-DL-ornithine, poly-L-ornithine or poly-δ-CBZ-DL-ornithine), and the like.

Polycationic species having a wide range of molecular weights can be employed in the practice of the present invention. Polycations having a MW in the range of about 200 up to 1,000,000 are suitable, with molecular weights in the range of about 1000 up to 100,000 preferred. Presently most preferred polycationic species for use in the practice of the present invention will have molecular weights in the range of about 5,000 to 50,000.

Optionally, the polycationic species employed in the practice of the present invention can be further modified with one or more functional groups capable of undergoing free radical polymerization. Suitable functional groups for this purpose include unsaturated species capable of free radical polymerization, such as, for example, acrylate groups, vinyl groups, methacrylate groups, and the like. When cells or tissues are treated with such modified polycationic species, the graft copolymer can be further subjected to free radical polymerization conditions, thereby stabilizing the association of graft copolymer with the cell surface. In addition, the further crosslinking of the graft copolymer forms a highly stabilized, immunoprotective coating of water-soluble polymer about the treated cell or tissue.

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Free radical polymerization of the above-described modified polycationic species can be carried out in a variety of ways, for example, initiated by irradiation with suitable wavelength electromagnetic radiation (e.g., visible or ultraviolet radiation) in the presence of a suitable photoinitiator, and optionally, cocatalyst and/or comonomer. Alternatively, free radical polymerization can be initiated by thermal decomposition of a suitable free radical catalyst, such as benzoyl peroxide, azobisisobutyronitrile, and the like.

Photoinitiators contemplated for use in the practice of the present invention include such ultraviolet (UV) initiators as 2,2-dimethyl phenoxyacetophenone, benzophenones and ionic derivatives (for water solubility), benzils and ionic derivatives thereof, thioxanthones and ionic derivatives thereof; and visible photoinitiators such as ethyl eosin, eosin, erythrosin, rose bengal, thionine, methylene blue, riboflavin, and the like.

Cocatalysts are typically used when the excited state of the photoinitiator is quenched too rapidly to efficiently promote polymerization. In such a situation, a cocatalyst (also referred to in the art as a "cosynergist", "activator", "initiating intermediate" or "quenching partner") will generally be employed. Cocatalysts contemplated for use in the practice of the present invention include triethanolamine, methyl diethanolamine, triethylamine, arginine, and the like.

Water-soluble polymeric species contemplated for use in the practice of the present invention are water-soluble polymers capable of rendering polycations non-immunogenic and include non-ionic, water-soluble polymers such as polyethylene glycol (PEG), polyvinyl alcohol (PVA), poly(hydroxyethyl methacrylate) (pHEMA), poly(acrylamide), poly(vinyl pyrrolidone) (PVP), poly(ethyl

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oxazoline), polysaccharides (such as, for example, starch, glycogen, guar gum, locust bean gum, dextran, levan, inulin, cyclodextran, agarose, and the like); as well as ionic, water-soluble polymers such as polyacrylic acid (PAA) or polysaccharides (such as, for example, xanthan gum, carageenan, hyaluronic acid, heparin, chitosan, pectin, and the like); as well as copolymers of any two or more of said water-soluble polymeric species. Presently preferred water soluble polymers employed in the practice of the present invention are polyalkylene oxides, such as polyethylene glycol (PEG).

Water-soluble polymeric species having a wide range of molecular weights can be employed in the practice of the present invention. Polymeric species having a MW in the range of about 200 up to 1,000,000 are suitable, with molecular weights in the range of about 500 up to 100,000 preferred. Presently most preferred polymeric species for use in the practice of the present invention will have molecular weights in the range of about 1000 to 50,000.

Optionally, the water-soluble polymeric species employed in the practice of the present invention can be further modified with one or more functional groups capable of undergoing free radical polymerization. Suitable functional groups for this purpose include unsaturated species capable of free radical polymerization, such as, for example, acrylate groups, vinyl groups, methacrylate groups, and the like. When cells or tissues are treated with such modified water-soluble polymeric species, the graft copolymer can be further subjected to free radical polymerization conditions, thereby stabilizing the association of graft copolymer with the cell surface. In addition, the further crosslinking of the graft copolymer forms a highly stabilized, immunoprotective coating of water-soluble polymer about the treated cell or tissue.

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Free radical polymerization of the above-described modified water-soluble polymeric species can be carried out in the same manner as described above with respect to free radical polymerization of modified polycationic species.

Graft copolymers contemplated for use in the practice of the present invention are those wherein the polycationic species has grafted thereon at least one water-soluble polymer chain per chain of said polycationic species, up to a maximum of one grafted chain per repeat unit of said polycationic species. For example, when the molecular weight of the polycationic species falls in the range of about 20,000, it will typically have grafted thereon at least about 5 chains of said water-soluble polymer chain per chain of polycationic species; with in the range of about 10-20 chains of said water-soluble polymer chain per chain of said polycationic species being the presently most preferred level of grafting. Those of skill in the art recognize that with polycationic species having higher molecular weights, higher levels of grafting will be desirable, and that the above values for grafting levels should be increased accordingly. Similarly, with respect to the water-soluble component of invention graft copolymers, the use of higher molecular weight species will allow one to achieve substantially the same result while grafting fewer (water-soluble) chains per chain of polycationic species.

Preparation of the graft copolymers of the present invention can be carried out employing chemical techniques known by those of skill in the art. For example, the free hydroxyl groups of the water-soluble component can be activated to render such groups susceptible to nucleophilic displacement. Thus, the free hydroxyl groups of the water-soluble component can be subjected to esterification, etherification, amidation,

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urethane formation, and the like. Such reactions involve the generation of such intermediates as carbonates, sulfonates, xanthates, epoxides, aliphatic aldehydes, carboxymethyl azides, succinimidyl succinates, and the like. The activated water-soluble component can then be coupled to a suitable polycationic species, for example, by nucleophilic displacement.

Cell types contemplated for use in the practice of the present invention include islets, fibroblasts, thyroid cells, parathyroid cells, adrenal cells, neuronal cells, dopamine secreting cells, hepatocytes, nerve growth factor secreting cells, adrenaline/angiotensin secreting cells, norepinephrine/metencephalin secreting cells, human T-lymphoblastoid cells sensitive to the cytopathic effects of HIV, and the like.

Also included within the scope of the present invention are cells having a modified cell surface which is substantially non-adhesive with respect to macromolecules encountered in physiological environments.

In accordance with another embodiment of the present invention, there is provided a process to remove copolymer compositions contemplated for use in the practice of the present invention from cells treated as described above, said process comprising contacting such cells with an effective amount of an anionic species.

Anionic species contemplated for use in this embodiment of the present invention include monomeric or polymeric anions. Any soluble anionic species capable of reversing the association of polycationic species with negatively charged cell surface can be employed for this purpose. Presently preferred anionic species are polyanionic species, such as, for example, heparin, heparin sulfate, chondroitin sulfate, soluble alginates (e.g.,

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sodium alginate, potassium alginate, ammonium alginate, and the like), bovine serum albumin, hyaluronic acid, pectin, carageenan, oxidized cellulose, and the like.

"Contacting" of treated cells to remove invention copolymer therefrom is carried out at room temperature for a time in the range of about 0.01 up to 1 hour or longer in physiological buffer solution containing anionic species at a sufficiently high ionic strength to reverse the association of polycationic species with negatively charged cell surface.

An effective amount of anionic species to employ in accordance with this embodiment of the present invention depends on the specific anionic species employed. Generally, the concentration of anionic species employed will be sufficient to reverse polycation binding to cells or tissues, but not so high as to be toxic to the biological material being treated. Concentrations employed are typically in excess of the amount of anion actually needed to disrupt binding of polycation to cell surface. Thus, for example, presently preferred treating solutions contain about 2.5 Units/ml of heparin or 2 mg/ml of bovine serum albumin.

In accordance with yet another embodiment of the present invention, there is provided a method to render cells non-immunogenic, said method comprising contacting said cells with an effective amount of a composition comprising a polycationic species having water-soluble polymer chains grafted thereon.

"Contacting" of cells with graft copolymer compositions to render cells non-immunogenic is typically carried out as described above with respect to rendering cells non-adhesive.

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The process of the present invention can be used for rendering non-immunogenic any cell, tissue, organ, or system of organs, and the like, that may be used for transplant or the like.

Also included within the scope of the present invention are cells having a modified cell surface which is substantially non-immunogenic with respect to mediators of immune response, e.g., biological macromolecules such as proteins, enzymes, and the like.

In accordance with another embodiment of the present invention, there is provided a process to remove copolymer compositions contemplated for use in the practice of the present invention from cells treated as described above, said process comprising contacting treated cells with an effective amount of an anionic species, as described above.

In accordance with still another embodiment of the present invention, there is provided a method to preserve cells and/or tissues which are subjected to long periods of storage before being used for therapeutic applications, said method comprising contacting said cells and/or tissues with an effective amount of a composition comprising a polycationic species having water-soluble polymer chains grafted thereon.

"Contacting" of cells and/or tissues with graft copolymer compositions to preserve same is typically carried out as described above with respect to rendering cells non-adhesive and/or non-immunogenic.

In accordance with a still further embodiment of the present invention, there is provided a method for associating water-soluble polymer with a cell surface, said method comprising:

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grafting water-soluble polymer onto a polycationic resin to produce a copolymer of said water-soluble polymer and said polycation, and thereafter

contacting said cell surface with an effective amount of said copolymer.

If desired, the copolymer can be substantially removed from the cell surface employing the above-described removal process.

In accordance with a further embodiment of the present invention, there is provided a method for the stabilization of liposomes having negatively charged surfaces, said method comprising contacting said liposomes with an effective, stabilizing amount of a composition comprising a polycationic species having water-soluble polymer chains grafted thereon.

"Contacting" of liposomes for the stabilization thereof is carried out at room temperature for a time in the range of about 0.01 up to 1 hour or longer in physiological buffer.

An effective amount of graft copolymer for use in this embodiment of the present invention is an amount sufficient to render such liposomes essentially non-detectable *in vivo*, thereby reducing uptake of the liposome by the reticuloendothelial system (and increasing liposome circulation times *in vivo*). Suitable quantities of graft copolymer will render the liposomes substantially non-adhesive to biological materials, while leaving the liposome intact, and without adversely affecting the function and/or activity of the contents thereof, if any. Typically, a concentration of graft copolymer sufficient to neutralize the negative charges on the surface of the liposome will be employed. Concentrations in the range of

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at least about 0.05% of graft copolymer, with respect to the concentration of the polycationic species used to treat the surface of said liposome will be employed; with concentrations of graft copolymer in the range of about 0.1 up to 0.5% being presently preferred.

One can readily determine the stability of a liposome using a functional assay, such as the following. In an *in vitro* setting, the stability of liposome-encapsulated hemoglobin in an un-modified liposome could be compared to the stability of hemoglobin encapsulated in a liposome stabilized in accordance with the present invention (i.e., the result of treating an un-modified liposome with a sufficient quantity of graft copolymer described above to stabilize the liposome). The release of hemoglobin into the surrounding buffer media over time would then be assayed, with an extended time-frame for release of hemoglobin indicating enhanced liposome stability.

In accordance with yet another embodiment of the present invention, there is provided a method for producing neural networks on a substrate, said method comprising:

masking that portion of said substrate which defines the desired network,

rendering the unmasked portion of said substrate non-adhesive by the above-described method of the invention,

removing the mask, then

allowing cells to spread and grow on said substrate, wherein cells grow only on the portion of the substrate which has not been treated with graft copolymer.

Substrates contemplated for use in the above-described method include tissue culture substrates, such as collagen, tissue culture polystyrene, microporous dextran substrate, and the like.

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Masking contemplated by the above-described method can be accomplished in a variety of ways, such as, for example, by covering a portion of the substrate with an agent which does not serve as a substrate for cell growth (e.g., a piece of tape, or the like).

The masking agent employed can readily be removed by merely reversing the process employed for applying the mask to the substrate.

Conditions required for cells to spread and grow on the substrate are standard cell culture conditions.

The resulting neural networks can be used for a variety of purposes, such as, for example, for studying the transmission of nerve impulses, for connection between a nerve cell and an electrical circuit, and the like.

The invention will now be described in greater detail by reference to the following non-limiting examples.

EXAMPLES

EXAMPLE I

Synthesis of a Graft copolymer of Poly-L-Lysine and Polyethylene Glycol

Twenty grams (20 g) of PEG (molecular weight 10,000 g/mol, having the structure HO-PEG-OH) were dried in a vacuum oven at 80°C for 24 hours and dissolved in 100 ml of methylene chloride that had been dried by molecular sieves (4A). Then, 3.24 g of 1,1-carbonyldiimidazole (CDI, 5 fold excess, to ensure the activation of 100% of PEG end groups) were added to the solution and stirred overnight at room temperature in an argon atmosphere. The CDI-activated PEG was then precipitated with an excess of anhydrous diethyl ether and dried overnight under vacuum. Five grams

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(5 g) of the CDI-activated PEG were dissolved in 20 ml of 5 mM sodium borate buffer (pH 9). In order to prevent crosslinking of poly-L-lysine (PLL) with the 100% CDI-activated PEG, 50% of the PEG end groups were inactivated by adding 30.2 μl of ethanolamine to the buffer solution and stirring for 4-6 hours at room temperature. This results in a mono-activated CDI-derivative of PEG, having the structure CDI-PEG-OH. Alternatively, a monomethoxy PEG could be used to avoid this partial deactivation step, but monomethyl PEGs are presently available commercially only up to molecular weight 5000.

Following the above-described partial deactivation step, 50 mg of PLL (M.W. 20,100 g/mol) were added to the reaction mixture and stirred for 24 hours at room temperature. The solution was then dialyzed for 24 hours against deionized water and freeze dried to obtain a powder. This procedure produced a PEG graft copolymer (PLL-PEG) having a concentration of approximately 10-20 PEG chains per PLL chain.

EXAMPLE II

Demonstration of Cell Binding Properties of PLL-PEG to Fibroblasts: Effect on Cells in Suspension

The cell binding effects of PLL-PEG copolymer produced as described in Example I were tested on cultures of human foreskin fibroblasts (HFF). These cells are anchorage dependent and ordinarily die within 4 to 10 hours if they do not adhere and spread on a surface. Thus, a flask of confluent HFF was harvested with trypsin-EDTA, then the resultant cells in suspension were split into 6 batches, each containing approximately 170,000 cells. Each batch was centrifuged to obtain cell pellets. Six different solutions were used for cell treatment:

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(A) Fibroblast culture media: Dulbecco's modified Eagles' medium (D-MEM) containing 10% fetal bovine serum;

(B) 10 mM HEPES buffered saline (HBS), pH 7.4;

(C) HBS containing 0.1% (w/v) PLL;

(D) HBS containing 0.5% PEG (M.W. 10,000);

(E) HBS containing 0.1% PLL and 0.5% PEG; and

(F) HBS containing 0.3% PLL-PEG (based on PLL concentration).

All solutions were sterilized by filtration through 0.22 micron filters prior to use. The cell pellets were resuspended in 2 ml of solutions A, B, C, D, E or F for approximately 10 minutes. The tubes were then centrifuged (200 xg for 5 minutes), the solutions aspirated and replaced with fibroblast culture medium, and the cells plated onto culture dishes. The plated cells were observed periodically to verify adherence and spreading. The cells were also stained with trypan blue (TB) to test viability. Table I summarizes the observations over 5 days following the seeding.

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TABLE I

HFF TREATMENT SOLUTIONS

TIME AFTER SEEDING	A (fibroblast culture medium)	B (buffered saline)	C (PLL)	D (PEG)	E (PLL + PEG)	F (PLL-PEG copolymer)
1 hr	Normal spreading	Normal spreading	No adherence; Cell clumping	Normal spreading	No adherence; Cell clumping	No adherence; No clumping
% viability	95	95	0	90	0	80
24 hr	Normal spreading	Normal spreading	No adherence; Cell clumping	Normal spreading	No adherence; Cell clumping	No adherence; No clumping
% viability	100	100	0	95	0	70

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TABLE I (cont)

HFF TREATMENT SOLUTIONS

TIME AFTER SEEDING	A (fibroblast culture medium)	B (buffered saline)	C (PLL)	D (PEG)	E (PLL + PEG)	F (PLL-PEG copolymer)
48 hr	Confluent monolayer	Confluent monolayer	No adherence; Cell clumping	Confluent monolayer	No adherence; Cell clumping	No adherence; No clumping
% viability	100	100	0	95	0	60
120 hr	Confluent monolayer	Confluent monolayer	No adherence; Cell clumping; Few spread Cells	Confluent monolayer	No adherence; Cell clumping; Few spread Cells	No adherence; No clumping; Few spread Cells
% viability	100	100	<1	95	<3	60

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Treatments A, B, and D showed essentially the same results, with most of the HFF showing normal spreading and viability.

Free PLL was found to be toxic at the concentrations used (treatments C and E). Essentially all cells subjected to treatments C and E took up TB and did not spread on the tissue culture substrate. The cells subjected to treatments C and E also showed extensive aggregation.

Free PEG had no appreciable effect on cell function (treatment D). PEG also had no appreciable ameliorating effect in conjunction with PLL (treatment E).

Incubation with the graft PLL-PEG copolymer of the present invention (treatment F) however, had a remarkable effect on the HFF. In stark contrast to treatment with free PLL, treatment with the copolymer PLL-PEG (at 3 times higher concentration than used for treatments C and E) produced cells that showed no adherence to the substrate, no aggregation in suspension, but a high percent viability. This viability was maintained for well over 24 hours with the HFF still in suspension. This behavior is quite unusual for anchorage dependent cells.

A distinct morphological difference in cells treated with PLL and PLL-PEG was evident. PLL treated cells in suspension showed a rough or ragged surface while those treated with PLL-PEG copolymer of the present invention are smooth and spherical, much like freshly trypsinized cells.

These results indicate that treatment with the PLL-PEG copolymer of the invention is noncytotoxic to HFF. In addition, interaction of the PEG-grafted polycation with the exterior of the cell prevents the cell from adhering to

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a substrate. Thus the cytotoxicity of PLL is markedly reduced by PEG grafting.

Five days after the initial treatment, a few of the cells treated with PLL-PEG copolymer begin to show some spreading on the surface of the culture dish. This observation implies that the PLL-PEG copolymer may either have desorbed from the cell surfaces, or cell division may have occurred (which would dilute the concentration of PLL-PEG copolymer on the cell membrane).

EXAMPLE III

Assessment of Efficacy of PLL and PLL-PEG Treatments at Various Dilutions

A similar experiment as outlined in Example II was conducted to test the effects of PLL and PLL-PEG copolymer at various dilutions. Solutions C and F were serially diluted with 10 mM HEPES buffered saline (HBS) to 1/5, 1/25, and 1/125 of their original concentrations, and human foreskin fibroblasts (HFF) incubated in these solutions for 10 minutes. Additional treatments included PEG 20M (a PEG composition having a molecular weight of about 20,000, comprised of two lower molecular weight PEGs (one having a MW ~8,000 and the other having a MW of ~10,000) linked together by a hydrophobic, bifunctional bisphenol-epichlorohydrin linker; available from Union Carbide, Danbury, CT) and PEG 20,000 (a substantially linear PEG having a molecular weight of ~20,000; available from Fluka, Ronkonkoma, NY) at 0.5% in HBS. A control treatment with fibroblast culture media was also run. Results are summarized in Table II, below.

In the Table, the following abbreviations are used:

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"adh." for adhesion,
"aggreg." for aggregated, and
"subst." is the abbreviation for substrate.

P-0 refers to cells treated with 0.1% of PLL, and P-5, P-25 and P-125 refer to cells treated with 1/5, 1/25, and 1/125 dilutions thereof, respectively. Similarly, G-0 refers to cells treated with 0.3% of PLL-PEG copolymer, and G-5, G-25 and G-125 refer to cells treated with 1/5, 1/25, and 1/125 dilutions thereof, respectively.

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TABLE II

HFF TREATMENT SOLUTIONS

TIME AFTER SEEDING	Control			PLL treatment				PLL-PEG copolymer treatment			
	No PEG	20M PEG	20,000 PEG	P-0	P-5	P-25	P-125	G-0	G-5	G-25	G-125
0 hr	------Minor adh. to substr.------			Clumped	Clumped	Clumped	Clumped; adh. to subst.	No adh.	No adh.	Minor adh. to subst.	Minor adh. to subst.
3 hr	---------100% adh. to subst.---------			Clumped; aggreg.; no adh. to subst.	Clumped; aggreg.; no adh. to subst.	Clumped; 5-10% adhered to subst.	Clumped; 5-10% adhered to subst.	No adh.	50% adh.	75% adh. to subst.	100% adh. to subst.
24 hr	---------100% adhesion---------			No adh; Extensive clumping	No adh; Extensive clumping	Clumped; ~10% adh. to subst.	Clumped; ~10% adh. to subst.	No adh.; no clumping	~60% adh.;	~80% adh.	~100% adhesion
% viability	---------100%---------			<10%	<10%	<10%	<10%	>70%	>70%	>70%	>70%

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Observation of the cells immediately after seeding showed all PLL treatments (abbreviated P) to cause clumping of cells. A small number of cells showed adherence in the P-125 treatment. The graft copolymer (PLL-PEG, abbreviated G) treatment showed a decrease in efficacy at the lower concentrations. At dilutions of 25 and 125 (G-25 and G-125), adherence of cells was noted, though not quantified. Treatments with the PEG 20M and PEG 20,000 showed no appreciable difference from the control.

Three hours following the initial seeding, the following observations were made. The PLL treated cells P-0 (0.1% PLL) and P-5 were clumped and aggregated, with none of the cells showing adherence to the substrate. P-25 and P-125 also showed clumping, but approximately 5-10% of cells adhered to the substrate, indicating PLL cytotoxicity at very low concentrations.

Cells treated with PLL-PEG showed an increased adhering tendency with increasing dilutions. G-0 (0.3% PLL-PEG) showed no adhesion and individual free-floating cells. G-5, G-25, and G-125 showed approximately 50%, 75% and 100% adherence, respectively, at 3 hours. G-125 was very similar to the PEGs and the control.

After 24 hours, P-0 and P-5 showed no adherence to substrate, and extensive clumping. P-25 and P-125 also showed clumping, but approximately 10% of the cells were adhered to the substrate, indicating a lower level of toxicity for P-25 and P-125, compared to the higher concentrations used in samples P-0 and P-5.

After 24 hours, G-0 showed no adherence to substrate and no clumping; while G-5, G-25 and G-125 showed increasing levels of adherence of approximately 60%, 80% and 100%, respectively. The PEG treatments and the control

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were also 100% adhered.

TB staining at 24 hours showed all PLL treatments to have less than 10% viability, while the treatments with PLL-PEG copolymer showed a viability of greater than 70%. Thus the attachment of PEG to PLL substantially alleviates the PLL toxicity; this effect is apparent at very low concentrations (P-125 = 0.0008% PLL; G-125 = 0.0024%).

EXAMPLE IV

Effect of PLL and PLL-PEG on Confluent Monolayers of Fibroblasts

In order to assess, in a more realistic (although *in vitro* situation), the effects of PLL and PLL-PEG copolymer of the invention on cells which would normally be present in a flattened spread morphology (and not in a rounded morphology), confluent monolayers of HFF were treated with solutions P-0, P-5, G-0, G-5, PEG 20M, and a control (fibroblast culture medium). The cells were exposed to these solutions for 10 minutes, followed by a rinse with HBS, then fibroblast culture medium was returned to the culture dishes.

Short-term observation 15 minutes after treatment showed the P-0 treated cells sloughing off the culture substrate, with approximately 90% of all cells in suspension at 20 minutes.

About 2-5% of cells treated with P-5 were detached from the surface within the same 15 minute period.

HFF treated with solutions G-0, G-5 and PEG 20M showed no appreciable difference from the control cells.

These results indicate that PLL (at 0.1%) is clearly toxic to HFF, while similar concentrations of PLL

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modified with PEG show no harmful effects to confluent monolayers of cells. It is noteworthy that the P-5 treatment showed only mild toxicity to spread, confluent fibroblasts, indicating that they may be less susceptible to toxic macromolecules in this state rather than in suspension.

EXAMPLE V

Reversal of PLL-PEG Binding to Cells with Polyanions

It was possible, by addition of heparin sulfate or chondroitin sulfate, to reverse the effect of PLL and PLL-PEG on HFF. Thus, addition of 2.5 U/ml of heparin to the fibroblast culture medium soon after treatment with PLL caused disaggregation of the HFF clumps and resulted in cells that were able to adhere to tissue culture substrates. If, however, the addition of heparin was postponed until several hours after the PLL treatment, reversibility was not possible because the cells had succumbed to PLL toxicity.

This however, was not the case with the PLL-PEG copolymer if the present invention. The nonadhesive, nonaggregating nature conferred upon the fibroblasts by treatment with PLL-PEG copolymer was found to be reversible at least 48 hours after the initial treatment, clearly indicating that these anchorage dependent cells were still alive, despite the fact that they were not adhered to a substrate.

EXAMPLE VI

Resistance of PLL-PEG Treated Cells to Specific Antibodies as Indicators of Conferred Immune Protection

Fibroblasts have receptors for the protein vitronectin on their surfaces. Vitronectin is a cell adhesion molecule (CAM). This receptor (called αV-ß3) can

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be targeted with an antibody, anti αV-ß3, a rabbit polyclonal. A fluorescently conjugated secondary antibody to anti αV-ß3 (e.g., rhodamine conjugated anti IgG, goat anti-rabbit) would permit the visualization of these receptors on the cell surface.

Untreated HFF, PLL treated HFF, and PLL-PEG treated HFF were incubated with anti αV-ß3 polyclonal antibody, followed by incubation with the secondary antibody, then observed at the appropriate excitation wavelengths under a microscope. It was observed that the untreated and PLL treated cells showed strong fluorescence, while the PLL-PEG treated cells fluoresced at a much lower level. This observation indicates that the approach of the antibody to the cell is hindered by the presence of PEG.

PLL by itself was found not to affect the receptor-ligand interaction.

Based on the above-described experiments, it is likely that the prevention of protein binding to these cells will render them immunologically unrecognizable.

EXAMPLE VII

Transplantation of PLL-PEG Treated Allogeneic Islets in Rats as a Model for Immunoprotectivity

Rat islets were isolated employing techniques known in the art [see, for example, Lacy and Kostianovsky in Diabetes 16:35 (1967)]. The isolated islets were treated with 0.3% PLL-PEG as described above (see Example II), and transplanted by injection into the peritoneal cavity of diabetic rats. Diabetes was induced by treatment with streptozotocin. Controls were injected with untreated rat islets. Blood glucose levels of these rats were monitored at first on an hourly basis, and then on a daily basis for several weeks. It was found that the control

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rats had a reversal of diabetes (indicated by normal glucose levels) for 3-4 days following which the graft failed due to rejection. On the other hand, the rats injected with the PLL-PEG copolymer treated islets showed a continuous reversal of diabetes for several weeks, indicating that the treatment of these cells with PLL-PEG copolymer was effective in immunoprotecting the islets.

EXAMPLE VIII

Crosslinkable Graft Copolymers

A variation on the above theme for the surface treatment of cells is one in which the PLL-PEG graft copolymer has on its structure polymerizable groups such as the acrylate group. The presence of this group on the graft copolymer facilitates polymerization or crosslinking following the absorption of the copolymer onto the cell surface through ionic interactions. The resultant covalently crosslinked network is significantly more stable than the ionically attached graft copolymer. Thus the immunoprotective properties conferred upon the cell by absorption of PLL-PEG on its surface are no longer transient as may be expected through an ionic interaction, but are permanent due to the formation of intermolecular and intramolecular covalent crosslinks formed with the PLL-PEG.

Synthesis of these polymerizable copolymers could have two possible strategies. One involves the synthesis of a PEG that is heterobifunctional, i.e., one end is functionalized with CDI (1,1-carbonyldiimidazole; or other electrophilic derivative) and the other with acryloyl chloride (the reaction of PEG with acryloyl chloride is described below). This technique allows the synthesis of a PLL-PEG graft copolymer in which the free end of PEG contains a polymerizable double bond. The second strategy involves the preparation of PLL-PEG as described above, and

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the subsequent reaction of the copolymer with acryloyl chloride to add polymerizable groups. In this case the addition of polymerizable groups to the copolymer is nonspecific, i.e., the substitution occurs on the free end of the PEG as well as on the amines on polylysine.

The reaction of PEG with acryloyl chloride proceeds to completion in about 24 hours when carried out at 50°C. For example, mono-CDI functionalized PEG (i.e., CDI-PEG-OH, prepared as described in Example I) was reacted with an equimolar amount of acryloyl chloride in dry dichloromethane solvent. The reaction was carried out in a round-bottom flask under an inert atmosphere at constant reflux for 24 hours. The resulting product was purified by precipitation with diethyl ether, then dried in a vacuum oven.

Alternatively, PLL-PEG could be treated with acryloyl chloride. In this situation, acrylate substitution would occur on both the PEG chains and the PLL backbone (via the amine groups thereof).

Photopolymerization is the method of choice for covalent crosslinking of the graft copolymer following attachment to the cell surface. Following attachment of the graft copolymer to the cell surface the treated cells are transferred to a physiological buffer solution containing ethyl eosin (EE, 0.1 μM to 0.1 mM), triethanolamine (TEA, 0.1 mM to 0.1 M), and optionally a comonomer (e.g. 1-vinyl 2-pyrrolidinone (VP) at a concentration in the range of about 0.001 to 1.0%, when used). This solution containing islets is well mixed and exposed to a mercury lamp (100 watt) with a bandpass filter (500-560 nm) for approximately 3 minutes. This causes crosslinking of the copolymer on the surface of the cell resulting in the immunoprotective layer. The cells are then transferred to culture.

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An alternative technique involves the incubation of the copolymer treated cells with a solution of EE (0.1 μM to 0.1 mM) in physiological buffer for approximately two minutes. In this step the EE complexes with the positively charged polycation on the cell surface. After rinsing in buffer the cells are transferred to a physiological buffer solution containing TEA (conc. as above), and a comonomer e.g. VP (optional). This solution containing islets is well mixed, polymerized as before, and transferred to culture.

EXAMPLE IX

PLL-PEG Solutions in Organ Preservation Media

As noted above, PEG 20M has been used in the preservation of organs. The basis of its activity, though not clearly understood, is believed to be the binding of PEG to cell surface molecules through nonspecific hydrophobic interactions. The PLL-PEG copolymer of the present invention, however, interacts directly through ionic interactions with cell-surface moieties bearing a negative charge. Thus, tissues and organs may be flushed with a solution containing the PLL-PEG copolymer prior to transplantation to, in effect, 'coat' the tissues with PEG, thereby providing an immunoprotective and organ-protective effect.

EXAMPLE X

Stabilization of Liposomes with PLL-PEG for Longer Circulation Times and Increased Biocompatibility

Lipid vesicles or liposomes have been investigated extensively as systems for drug delivery (Gregoriadis, 1987). The commonly used phospholipids that comprise liposomes, such as phosphatidyl choline, phosphatidyl serine, dilaurylphosphatidic acid, and phosphatidylglycerol are negatively charged at

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physiological pH. The interaction of polycations such as PLL with the negatively charged phospholipids has been studied quite extensively with regard to conformational changes induced in PLL and consequent stability [Fukushima et al., *Biophysical Chemistry* 34:83 (1989); Houbre et al., *Biophysical Chemistry* 30:245 (1988)]. Stability of liposomes in physiological conditions is a major focus of researchers investigating drug delivery. Although PLL may be used to stabilize lysosomes *in vitro*, PLL coated liposomes *in vivo* are likely to be rapidly overgrown or ingested by macrophages due to the adhesive nature of PLL, thus making them ineffective for the controlled release of drugs. In addition, liposomes may also be destroyed by uptake by the reticuloendothelial system. The addition of the graft copolymers of the present invention to the surface of the liposome is likely to prevent this uptake.

The replacement of PLL by the PLL-PEG copolymer of the present invention, however, promises to provide a liposome that is stable not only due to interactions between negatively charged phospholipid and positively charged PLL, but also because the PLL-PEG copolymer will prevent interactions with proteins, and therefore prevent interactions with cells such as macrophages. This should result in liposomes with long circulation times which can therefore deliver drugs in a controlled fashion.

EXAMPLE XI

Patterned Surfaces for Neural Networks

Investigators in neurology have tried to generate *in vitro* networks of neurons on culture dishes. A problem has been to generate patterned surfaces that are preferentially adherent to cells in order to design 'biological circuits.' By creating a mask of the pattern desired, and applying it to the culture substrate, followed by treatment of the surface with PLL-PEG copolymer, one can

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selectively leave the desired pattern adhesive to cells, while the rest of the available surface is rendered nonadhesive to cells.

While the invention has been described in detail with reference to certain preferred embodiments thereof, it will be understood that modifications and variations are within the spirit and scope of that which is described and claimed.

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That which is claimed is:

1. A method to render cells non-adhesive, said method comprising contacting said cells with an effective amount of a composition comprising a polycationic species having water-soluble polymer chains grafted thereon.

2. A method according to claim 1 wherein said water-soluble polymer is selected from polyethylene glycol (PEG), polyvinyl alcohol (PVA), poly(hydroxyethyl methacrylate) (pHEMA), polyacrylic acid (PAA), poly(acrylamide), poly(vinyl pyrrolidone) (PVP), poly(ethyl oxazoline) (PEOX), polysaccharides, or copolymers of any two or more thereof.

3. A method according to claim 1 wherein said polycationic species has grafted thereon at least one water-soluble polymer chain per chain of said polycationic species.

4. A method according to claim 3 wherein said water-soluble polymer is polyethylene glycol.

5. A method according to claim 1 wherein either the water-soluble polymer, or the polycationic species, or both contain at least one functional group which is susceptible to free radical polymerization.

6. A method according to claim 5 wherein said composition is further subjected to free radical polymerization conditions.

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7. A method according to claim 1 wherein said polycationic species is selected from:

polyethyleneimine, polyallylamine, polyetheramine, polyvinylpyridine,

polysaccharides having a positively charged functionality thereon,

polyamino acids selected from:
poly-L-histidine, poly-im-benzyl-L-histidine,
poly-D-lysine, poly-DL-lysine, poly-L-lysine,
poly-ϵ-CBZ-D-lysine, poly-ϵ-CBZ-DL-lysine,
poly-ϵ-CBZ-L-lysine,

poly-DL-ornithine, poly-L-ornithine,
poly-δ-CBZ-DL-ornithine,

poly-L-arginine,

poly-DL-alanine-poly-L-lysine;

poly(-L-histidine, L-glutamic acid)-poly-DL-alanine-poly-L-lysine;

poly(L-phenylalanine, L-glutamic acid)-poly-DL-alanine-poly-L-lysine; or

poly(L-tyrosine, L-glutamic acid)-poly-DL-alanine-poly-L-lysine;

random copolymers of:
L-arginine with tryptophan, tyrosine, or serine;
D-glutamic acid with D-lysine; or
L-glutamic acid with lysine, ornithine, or mixtures thereof;

as well as mixtures of any two or more thereof.

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8. A method according to claim 1 wherein said polycationic species is selected from polylysine or polyornithine.

9. A method according to claim 1 wherein said cells to be rendered non-adhesive are selected from islets, thyroid cells, adrenal cells, dopamine secreting cells, hepatocytes, or human T-lymphoblastoid cells sensitive to the cytopathic effects of HIV.

10. The cellular product obtained by the method of claim 1.

11. A process to remove composition(s) comprising a polycationic species having water-soluble polymer chains grafted thereon from cells treated in accordance with the method of claim 1, said process comprising contacting said treated cells with an effective amount of an anionic species.

12. A process according to claim 11 wherein said anionic species is monomeric or polymeric.

13. A process according to claim 12 wherein said anionic species is a polyionic species selected from heparin, heparin sulfate, chondroitin sulfate, bovine serum albumin, soluble alginates, hyaluronic acid, pectin, carageenan, or oxidized cellulose.

14. Cells having a modified cell surface which is non-adhesive with respect to mediators of immune response, wherein the surface of said cells have been modified with a composition comprising a polycationic species having water-soluble polymer chains grafted thereon.

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15. A method to render cells non-immunogenic, said method comprising contacting said cells with a composition comprising a polycationic species having water-soluble polymer chains grafted thereon.

16. A method according to claim 15 wherein said polycationic species has grafted thereon at least one water-soluble polymer chain per chain of said polycationic species.

17. A method according to claim 16 wherein said water-soluble polymer is polyethylene glycol.

18. A method according to claim 15 wherein said cells to be rendered non-immunogenic are selected from islets, thyroid cells, adrenal cells, dopamine secreting cells, hepatocytes, or human T-lymphoblastoid cells sensitive to the cytopathic effects of HIV.

19. The cellular product obtained by the method of claim 15.

20. A process to remove composition comprising polycationic species having water-soluble polymer chains grafted thereon from cells treated in accordance with the method of claim 15, said process comprising contacting said treated cells with an effective amount of an anionic species.

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21. A method to preserve cells and/or tissues which are subjected to long periods of storage before being used for therapeutic applications, said method comprising contacting said cells and/or tissues with an effective amount of a composition comprising a polycationic species having water-soluble polymer chains grafted thereon.

22. A method for associating water-soluble polymer with a cell surface, said method comprising:

grafting water-soluble polymer onto a polycationic resin to produce a copolymer of said water-soluble polymer and said polycation, and thereafter

contacting said cell surface with an effective amount of said copolymer.

23. A method according to claim 22 wherein said copolymer comprises a polycation having grafted thereon at least one water-soluble polymer chain per chain of said polycationic resin.

24. A method for the stabilization of liposomes having negatively charged surfaces, said method comprising contacting said liposomes with an effective, stabilizing amount of a composition comprising a polycationic species having water-soluble polymer chains grafted thereon.

25. A method according to claim 24 wherein said polycationic species has grafted thereon at least one water-soluble polymer chain per chain of said polycationic species.

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26. A method for producing neural networks on a substrate, said method comprising:

masking that portion of said substrate which defines the desired network,

rendering the unmasked portion of said substrate non-adhesive by the method of claim 1,

removing the mask, then

allowing cells to spread and grow on said substrate.

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INTERNATIONAL SEARCH REPORT

International application No.
PCT/US93/02609

A. CLASSIFICATION OF SUBJECT MATTER

IPC(5) :A01N 1/02; C12P 1/00; C12N 5/00
US CL :435/1, 41, 240.1, 240.22, 962
According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 435/1, 41, 240.1, 240.22, 962

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

CAS ONLINE, APS, BIOSIS, EMBASE, MEDLINE

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	WO, A, 91/07951 (Skjak-Braek et al.) 13 June 1991, see page 3 and claim 10.	1-10
Y	Neuroscience Letters, Volume 49, issued 1984, U. T. Ruegg et al., "Growth of Dissociated Neurons in Culture Dishes Coated with Synthetic Polymeric Amines," pages 319-324, see page 319.	1-10
Y	Transplantation, Volume 51, No. 1, issued January 1991, Tianli Zheng et al., "Prolonged Pancrease Preservation Using A Simplfied UW Solution Containing Polytheylene Glycol", pages 63-66, see page 63, abstract.	1-10

[X] Further documents are listed in the continuation of Box C. [ ] See patent family annex.

* Special categories of cited documents:

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Date of the actual completion of the international search

12 May 1993

Date of mailing of the international search report

08 JUN 1993

Name and mailing address of the ISA/US
Commissioner of Patents and Trademarks
Box PCT
Washington, D.C. 20231

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INTERNATIONAL SEARCH REPORT

International application No.
PCT/US93/02609

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	Journal of Biomedical Materials Research, Volume 25, issued 1991, Neil P. Desai et al., "Biological Responses to Polyethylene Oxide Modified Polyethylene Terephthalate Surfaces",pages 829-843, see page 829, abstract.	1-10

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INTERNATIONAL SEARCH REPORT

International application No.
PCT/US93/02609

Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This international report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☐ Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:

2. ☐ Claims Nos.:
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:

3. ☐ Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:
(Form PCT/ISA/206 Previously Mailed.)
Please See Extra Sheet.

1. ☐ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.

2. ☐ As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.

3. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:

4. ☒ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:
1-10

Remark on Protest

☐ The additional search fees were accompanied by the applicant's protest.

☐ No protest accompanied the payment of additional search fees.

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INTERNATIONAL SEARCH REPORT

International application No.
PCT/US93/02609

BOX II. OBSERVATIONS WHERE UNITY OF INVENTION WAS LACKING
This ISA found multiple inventions as follows:

I. Claims 1-10, drawn to a method of making non-adhesive cells and the product of the method.
II. Claims 11-13, drawn to a process to remove compositions.
III. Claim 14, drawn to cells with modified immune response.
IV. Claims 15-19, drawn to a process to make cells non-immunogenic and the product of the process.
V. Claim 20, drawn to a process to reverse non-immunogenic cells.
VI. Claim 21, drawn to a method of storage.
VII. Claims 22-23, drawn to a method of applying.
VIII. Claims 24-25, drawn to a method of stabilizing liposomes.
IX. Claim 26, drawn to a method for making neural networks.

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